418 Rec'd PCT/PTO 23 DEC 1999

FORM PTO-1390 (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER:

U.S. APPLICATION NO. (If known, see 37 C.F.R.1 5): 09/446681

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB98/01893	29 June 1998 (29.06.98)	27 June 1997 (27.06.97)

TITLE OF INVENTION: BIOSENSOR MATERIALS AND METHODS.

APPLICANT(S) FOR DO/EO/US: ARCHER, John Anthony Charles et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. X ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. X ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. X ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. X ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. X ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. X ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. X ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. X ☒ Other items or information:
    1. Certificate of Express Mailing By Express Mail Under 37 CFR 1.10

416 Rec'd PCT/PTO 23 DEC 1999

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/446681	PCT/GB98/01893	

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO..... **$830.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) **$640.00**		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).. **$710.00**		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37CFR 1.445(a)(2)) paid to USPTO..... **$950.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)..... **$90.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840	00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130	00

Claims	Number Filed	Number Extra	Rate		
Total Claims	49 -20 =	29	X18.00	$ 522	00
Independent Claims	2 -3 =	0	X78.00	$ 0	00
Multiple dependent claims(s) (if applicable)			+ $230.00	$ 0	00
TOTAL OF ABOVE CALCULATIONS =				$ 1492.	00
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	-
SUBTOTAL =				$ 1492	00
Processing fee of **$130.00** for furnishing the English translation later the ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ 0	00
TOTAL NATIONAL FEE =				$ 1492	00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 0	00
TOTAL FEES ENCLOSED =				$ 1492	00
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ 1492.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-1406. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Patrick J. Hagan, Esq.
DANN, DORFMAN, HERRELL AND SKILLMAN
1601 Market Street
Suite 720
Philadelphia, PA 19103-2307

Patrick J. Hagan
SIGNATURE

Patrick J. Hagan
NAME

27,643
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of)
)
John Anthony Charles ARCHER et al.)
)
U.S. Application No.: Not Yet Assigned)
[Int'l Application No. PCT/GB98/01893])
)
Filed: Concurrently Herewith)
[Int'l Filing Date: 29 June 1998))
)
For: BIOSENSOR MATERIALS AND METHODS)

PRELIMINARY AMENDMENT

Before calculation of the filing fee, please amend the claims of the above-referenced patent application, which claims are based on the Article 34 claim amendments filed in the corresponding international patent application, as follows:

Claims 4, 5, 7, 9, and 10, line 1 of each claim, delete "any one of the preceding claims" and insert - - claim 1 - -;

Claim 6, line 1, delete "any one of the preceding claims" and insert - - claim 1 - -;

line 2, delete "screen is performed" and insert - - determined - -;

Claims 12 and 13, line 1 of each claim, delete "any one of claims 1 to 9" and insert - - claim 1 - -;

Claim 16, line 1, delete "any one of claims 13 to 15" and insert - - claim 1 - -;

Claim 19, line 1, delete "any one of claims 16 to 18" and insert - - claim 16 - -;

Claim 20, line 1, delete "any one of claims 16 to 19" and insert - - claim 16 - -;

Claim 24, line 1, delete "any one of claims 21 to 23" and insert - - claim 21 - -;

Claim 25, lines 4 and 5, delete "any of the preceding claims" and insert

- - claim 1 - -;

Claim 26, lines 3 and 4, delete "any one of claims 1 to 24" and insert - - claim 1 - -;

Claim 29, line 4, delete "or claim 27";

Claim 30, line 1, delete "any one of claims 26 to 29" and insert - - claim 26 - -;

Claim 31, line 5, delete "any one of claims 1 to 24" and insert - - claim 1 - -;

Claim 32, lines 1 and 2, delete "or claim 31";

Claim 35, line 2, delete "any one of claims 32 to 34" and insert - - claim 32 - -;

Claim 37, line 1, delete "or claim 36";

Claim 38, line 2, delete "any one of claims 35 to 37" and insert - - claim 35 - -;

Claim 39, line 2, delete "any one of claims 32 to 34" and insert - - claim 32- -;

Claim 42, line 1, delete "or claim 41";

Claim 43, line 1, delete "any one of claims 40 to 42" and insert - - claim 40 - -;

Claim 44, lines 1 and 2, delete "any one of claims 40 to 43" and insert - - claim 40 - -;

Claim 45, lines 1 and 2, delete "any one of claims 1 to 24" and insert - - claim 1 - -;

lines 4 and 5, delete "any one of claims 21 to 24. 18 August 1999" and insert - - claim 21 - -

Please add the following new claims:

46. A nucleic acid comprising a sequence encoding a modified inducible promoter obtainable by the method of claim 25 which is at least 70%; 80%; 90%; 95% or 98% identical to the sequence of the inducible promoter of claim 27.

47. A vector comprising the nucleic acid of claim 31.

48. A method as claimed in claim 36 wherein the host cell is a mycolic acid bacterium of the same strain from which the inducible promoter and/or operon proteins were isolated.

49. A method as claimed in claim 41 wherein the signal is detected by an increased expression of a heterologous signal protein from a signal gene.

REMARKS

The purpose of this Preliminary Amendment is to delete multiple claim dependencies.

Favorable consideration of the present application is respectfully requested.

Respectfully submitted,

DANN, DORFMAN, HERRELL AND SKILLMAN
A Professional Corporation

By Patrick J. Hagan
PATRICK J. HAGAN
PTO Registration No. 27,643

Telephone: (215) 563-4100

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant or Patentee: John Anthony Charles ARCHER, David Keith SUMMERS, Herve Jacquieu ROLAND and Justin Antoine Christian POWELL
Application or Patent No.: 09/446,681
Filed or Issued: December 23, 1999
For: BIOSENSOR MATERIALS AND METHODS

VERIFIED STATEMENT (DECLARATION) FOR SMALL ENTITY STATUS [37 CFR §1.9(f) and §1.27(c)] - SMALL BUSINESS CONCERN

I hereby declare that I am making this verified statement to support a claim by the above-identified applicant or patentee for small entity stat for purposes of paying reduced fees with regard to the above-identified invention described in

[ ] the specification filed herewith
[X] U.S. Patent Application No. 09/446,681, filed December 23, 1999
[ ] U.S. Patent No. _____, issued _____

I hereby declare that I am empowered to act on behalf of the small business concern identified below:

X [ ] I am the owner.
[ ] I am empowered to act as Director of the concern.

Full name of the concern: Cambridge University Technical Services Limited
Address of the concern: The Old School, Cambridge CB2 1TS, Great Britain

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR §121.3-18, an reproduced in 37 CFR §1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to contro both.

I hereby declare that rights under contract or law have been conveyed to and remain with the above-identified small business concern with regard to the above-identified invention.

If the rights held by the small business concern are not exclusive, each individual, concern or organization known to have rights to the invention is listed below* and the concern knows of no rights to the invention being held by any person, other than the inventor, who could n qualify as an independent inventor under 37 CFR §1.9(c) if that person had made the invention, or by any concern which would not qualify a a small business concern under 37 CFR §1.9(d) or by a nonprofit organization under 37 CFR §1.9(e).

FULL NAME:
ADDRESS:

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION
FULL NAME:
ADDRESS:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION
FULL NAME:
ADDRESS:
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION
*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention aver to their status as small entities. (37 CFR §1.27)

I acknowledge the duty to file, in this application or patent, notification of any changes in status resulting in loss of entitlement to small entit status prior to paying, or at the time of paying the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR §1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: X DR. RICHARD C. JENNINGS
Title in Organization : X DIRECTOR
Address : The Old School, Cambridge CB2 1TS, Great Britain
Signature : X R. C. Jennings **Date:** 21 February 2000

BIOSENSOR MATERIALS AND METHODS

Technical Field

This invention relates to biosensor materials and methods, and in particular to methods for generating microorganisms having utility in biosensing, tools which can be generally used in such methods, the microorganisms themselves, and biosensing methods employing such microorganisms.

Background Art

It is frequently desirable to be able to detect small concentrations of analytes in samples, e.g. environmental samples. For instance, to allow more effective management of scarce environmental resources, more efficient and faster methods of assessing environmental pollution are required. At present, molecular-specific monitoring of effluent streams and other environmental matrices requires extensive chemical manipulation of the sample followed by Gas Chromatography (GC) and Mass Spectrometry (MS) analyses. Although these techniques are highly sensitive, sample preparation is necessarily slow and expensive. Consequently, continuous on-site analysis of a variety of environmental matrices cannot be achieved using these methods at reasonable cost.

An alternative method for the determination of phenols and chlorophenols has been proposed using a biosensor based around *Rhodococcus* sp. [see Riedel et al (1993) Appl Microbiol Biotechnol 38: 556-559]. In this method microorganisms are immobilised in an oxygen electrode, and oxygen uptake in response to added substrates was monitored. Although fairly simple and rapid, this method lacks robustness and is not sufficiently sensitive or specific for detecting particular environmental pollutants.

It can thus be seen that the provision of novel

materials and methods capable of being used in the field of biosensing would represent a step forward in the art.

Disclosure of Invention

In a first aspect of the invention there is disclosed a method of detecting the presence or absence of an analyte in a sample comprising the steps of:
(a) contacting the sample with a transformed microorganism which is a mycolic acid bacterium which expresses a binding agent capable of binding the analyte, wherein the binding of the agent to the analyte causes a detectable signal, and wherein said bacterium has been transformed such as to improve the detectability of the signal, and
(b) observing said bacterium for said detectable signal;

By "observing" is meant ascertaining by any means (directly or indirectly) the presence or absence of the selected signal which is indicative of the binding event.

By "improve" is meant, inter alia, altering the nature of the signal to one which can be observed more readily or increasing the intensity of the signal (thereby reducing the sensitivity of the means used to observe it).

Thus by using a transformed microorganism, the limitations inherent in wild-type microorganisms such as those used in the prior art may be overcome. In particular more sensitive and robust monitoring methods than those based on natural biochemical activities such as oxygen uptake can be employed. The mycolic acid bacterial gene expression-based sensors of the present invention can combine high sensitivity with the biofiltering and bioconcentrating aspects of the mycolic acid bacterial cell wall. Methods for generating such transformants are described in further detail below. Such transformed microorganisms are hereinafter referred to as 'biosensors'.

Preferably the analyte is an environmental

pollutant, for instance such as may result from industrial or medical applications. Of particular interest is the detection of mono- and poly-aromatic, cyclic, heterocyclic and linear hydrocarbons such as, but not limited to, components of fuels, solvents, propellants, energetics and pesticides (such as may appear on United States EPA Priority Pollutants List and European Community Grey and Black Lists) and naturally occurring degradation products of these compounds in industrial process media, vapours, effluents, raw water, rivers, ground waters, or soils. As will be clear to the skilled person from the disclosure hereinafter, the methodology of invention is inherently flexible and may, in principle, be employed to develop mycolic bacteria capable of biosensing almost any target analyte.

The mycolic acid bacteria form a supra generic group of Gram-positive, non-sporulating bacteria which is comprised of the genera Corynebacterium, Mycobacterium, Nocardia, Rhodococcus, Gordona, Dietzia and Tsukamurella. Members are metabolically diverse and capable of using as sole carbon source (a growth-inducing substrate) a wide range of natural and xenobiotic compounds, including many key environmentally-toxic and/or industrially-important molecules e.g. hydrophic organic compounds. The mycolic acid bacteria exhibit several structural and physiological features which appear to be specialisations for hydrocarbon degradation, these include a hydrophobic mycolic acid outer cell layer and associated production of extracellular mycolic acid-derived biosurfactants. Most preferably the bacterium is a member of the Rhodococcus or Nocardia complex (i.e. nocardioform actinomycete).

The detectable signal may be a change in enzyme function(s), metabolic function(s) or gene expression.

Preferably however the signal is ascertained in consequence to an increased expression of a signal protein from a signal gene, more preferably a

heterologous signal gene. Many suitable signal proteins (which have a readily detectable activity) are known in the art e.g. ßgalactosidase, which can generate a coloured substrate. The signal may utilise co-factors. Most preferably the activity of the signal protein, or the protein itself, can be estimated photometrically (especially by fluorimetry). This may be directly e.g. using instance green (and red) fluorescent protein, insect luciferase, and photobacterial luciferase. Alternatively it may be indirect e.g. whereby the signal gene causes a change which is detected by a colour indicator e.g. a pH change. Methods for introducing signal genes into appropriate hosts are described in further detail below.

Generally the bound agent/analyte complex will initiate expression of a signal gene which is operably linked to an inducible promoter. The identification of suitable promoters and/or coding sequences which are operably linked to them (including that of the binding protein) in mycolic acid bacteria, in order to modify said suitable promoters and/or coding sequences to introduce signal genes therein forms one part of the present invention.

As used herein, "promoter" refers to a non-coding region of DNA involved in binding of RNA polymerase and other factors that initiate or modulate transcription from a coding region of DNA whereby an RNA transcript is produced.

An "inducible" promoter requires specific signals in order for it to be turned on or off.

The terms "operatively linked" and "operably linked" refer to the linkage of a promoter to an RNA-encoding DNA sequence, and especially to the ability of the promoter to induce production of RNA transcripts corresponding to the DNA sequence when the promoter or regulatory sequence is recognized by a suitable polymerase. The term means that linked DNA sequences (e.g., promoter(s), structural

gene (e.g., reporter gene(s)), terminator sequence(s), are operational or functional, i.e. work for their intended purposes.

As is known to those skilled in the art, the transport and binding proteins (agents) required for the functionality of the inducible promoter, as well as the catabolic enzymes induced by it, will frequently form part the operon containing the promoter, and may thus be identified and isolated along side it using the methods disclosed above. These additional proteins are hereinafter referred to as "operon proteins".

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression in common hosts such as E. coli. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al,* 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

However, the present inventors have recognised that certain methods previously employed in the art which were developed for enteric bacteria such as E. coli may not be the most appropriate for use in mycolic acid bacteria. The mycolic acid layer and associated biosurfactants (which are a defining feature of these bacteria) and thick cell wall confer great resistance to cell lysis

protocols known in the art. Similarly, mycolic strains used in the invention may not (indeed generally will not) be laboratory type strains, and may thus exhibit very high levels of nuclease activity.

In addition the detailed chemistry of the inducible pathway which forms the basis of the biosensors of the present invention will frequently not be known e.g. if there are no known enzyme pathways leading to the degradation of a particular analyte, or possibly the analyte is not mineralised completely and is only partially utilised in an uncharacterised but inducible pathway. Therefore cloning by acquisition of some defined enzyme activity, assayed through a particular reaction (as opposed to a general phenotypic activity which results in gain of utilisation of a particular analyte as a source of metabolically useful products) may not be a plausible option to isolate genes from a wild type mycolic acid bacterium.

Accordingly, advantageous methods have been developed by the inventors which in preferred forms allow the rapid isolation and characterisation of promoters and operably linked operon proteins which avoid or at least minimise host restriction and requires no prior knowledge of the inducible enzyme chemistry involved. The methods of identifying, modifying and employing novel inducible promoters and/or coding regions operably linked to them which are appropriate to mycolic acid bacteria are detailed below.

Thus in a second aspect of the invention there is disclosed a method for identifying DNA encoding an inducible promoter which is induced in response to a specific analyte and/or identifying DNA encoding associated operon proteins comprising the steps of:

(a) culturing a source of mycolic acid bacteria in a selective medium containing said specific analyte and being selective for oligotrophic bacteria,

(b) identifying bacteria capable of subsisting on said

medium,

(c) extracting DNA from said bacteria

(d) incorporating said DNA into vectors

(e) cloning said vectors into a suitable host cells

(f) screening the host cells for said inducible promoter and/or proteins in order to identify vectors encoding it.

By "screening" is meant subjected to analysis in order to determine the presence or absence of a particular defined property or constituent. Generally, in order to construct a biosensor strain against a particular analyte, isolation *de novo* from the soil or other environmental matrices of mycolic acid bacteria which exhibit inducible expression of catabolic genes in the presence of the analyte will be required. Methods of screening are discussed in more detail below.

As is known to those skilled in the art "oligotrophic bacteria" are bacteria which exhibit a preference for, and persistent slow growth on, very low levels of bioavailable carbon sources. These bacteria are adapted to and predominate in carbon-poor environments (predominantly aquatic habitats where carbon is limiting to μM levels). The term as used herein is intended also to embrace those bacteria which are capable of growing on defined minimal media without supplementary amino acids and vitamins (sometime termed prototrophic). Such bacteria are rarely capable of the very rapid growth as exemplified by the enteric bacterium E. coli, but are by contrast, extremely persistent and metabolically versatile. Work done by the present inventors has shown that, generally speaking, auxotrophic bacteria are not suitable as biosensor strains for environmental and industrial use.

Preferably the medium used in the second aspect is a defined minimal medium called hereinafter 'MMRN' which has been developed by the present inventors to screen for the oligotrophic mycolic acid bacteria (especially rhodococcal and nocardial strains) which form the basis

of the biosensor. This medium preparation is a derivative of von der Osten et al.(1989) but for mycolic acid bacteria sodium citrate and biotin are omitted. Most importantly, the level of carbon supplement is reduced to oligotrophic levels (<500 μM, more preferably <100 μM). Experiments show that MMRN facilitates simple, selective enrichment for oligotrophic, mycolic acid-containing bacteria as well as providing the basis for testing and characterisation of gene induction. The medium forms a third aspect of the present invention.

DNA may be extracted from the bacteria by any methods known in the art. However, the present inventors have demonstrated that DNA isolation from mycolic acid soil bacteria (particularly novel isolates which are generally highly resistant to lysis) using standard techniques is inefficient. Accordingly, several optimised methods of generating total DNA from mycolic bacteria have been developed, as described in more detail below (Examples 3 and 4). These involve bacterial culture in MMRN supplemented with L-glycine, oligotrophic levels of carbon source (80 μM) and removal of biosurfactants by washing in a non-ionic detergent (e.g. Tween 80) prior to a modified alkaline lysis technique. The concept of using a non-ionic detergent at between 0.05 - 0.5 % (preferably 0.1%) in order to facilitate DNA extraction is central to the novel, optimised methods.

"Vector", unless further specified, is defined to include, inter alia, any plasmid DNA, lysogenic phage DNA and/or transposon DNA, in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

Preferably the host used is E. coli. More preferably it is an E. coli strain carrying one or more of the

mcrABC mrr hsdSRM recA and recO mutations, since this is believed to enhance clone recovery when using DNA derived from mycolic acid bacteria which (e.g. in Rhodococcus/Nocardia) is GC rich,. Gene libraries may be readily maintained in these strains.

Preferably the vector used with E. coli further incorporates the 'cos' element (which is well known to those skilled in the art). Because of their capacity and selection for large DNA inserts and efficient transfection rates, cosmid cloning vectors facilitate rapid gene library construction, which is especially useful in the present context because the activities of interest are often encoded by closely lined genes or operons which may be contained on relatively large fragments of the e.g. Rhodococcus/Nocardia genome.

Preferably the mycolic acid bacteria isolates are further screened, for instance after stage (b), to ensure an absence of catabolic repression. Catabolite repression is the selective control of gene expression in response to the energy state of the cell. This process is part of a range of gene expression strategies grouped under the "stringent/relaxed" responses. Together, these allow bacteria to optimise their metabolism for maximum energy efficiency. At the genetic level, catabolite repression is achieved by the selective expression of one of several sigma factors, each expressed under a different physiological state and/or growth phase (Fujita *et al*, 1994) each recognising a different promoter sequence (Bashyam *et al*, 1996). This facilitates the selective expression or repression of a wide range of genes and operons simultaneously via the regulation of a single gene product.

To create an efficient, functional biosensor, such media-associated repression/activation phenomena must be absent or be disabled in the host strain since, in principle, catabolite repression could seriously compromise the activity of a biosensor because the

presence of a more efficient carbon source (such as glucose, succinate or acetate etc.) would lead to repression of hydrocarbon catabolic pathways which forms the basis of the sensor. Mycolic acid bacteria Brevibacterium (Oguiza *et al.*, 1996), Corynebacterium, Nocardia (Takahashi, *et al*, 1991), Mycobacterium smegmatis, M. Tuberculosis, and M. bovis BCG (Bashyam *et al*, 1996) and M. leprae (Doukhan *et al*, 1995) encode multiple sigma factor genes consistent with global stringent/relaxed genetic control. Consistent with these data, catabolite repression has been experimentally observed in Rhodococcus (Baryshnikova, *et al*, 1997).

To identify strains lacking catabolic repression, the concentrations of an enzyme known to be, or suspected of being, associated with the catabolic pathway of interest (e.g. catechol 2,3-dioxygenase, which is associated with toluene catabolism) is assessed in (a) selective medium supplemented with the specific analyte, (b) selective medium supplemented with the specific analyte plus a high efficiency carbon source such as glucose (1 mM) and (c) selective medium supplemented with glucose (1 mM) alone. Enzyme activities should be very low or undetectable in the absence of analyte. In the presence of analyte, and glucose plus analyte, the activities should be, within experimental error, very similar. To ensure that not only are biosensor strains free from all complex media-associated repression/activation effects, microbiological screenings are preferably extended to include several complex media. e.g. Lauria Bertini broth or Nutrient Agar in addition to MMR + 1mM levels of individual carbon sources.

The present inventors have established that catabolic genes in mycolic acid bacteria exhibit poor DNA sequence conservation with analogous enzyme genes in Gram negative bacteria. As a result, "reverse genetic" approaches to isolation of novel catabolic pathways are likely to be of limited use when using such published

sequence data.

Thus in one embodiment of the second aspect, the host cells are screened for the inducible promoter and/or operon proteins by screening the cells using one or more probes based on the sequence of other promoters and/or operon proteins employed by mycolic acid bacteria in catabolic enzyme production. One example of a source of suitable sequences is the promoter operator region of the R. corallina orthohydroxyphenylpropionic - ohp - acid catabolic operon (which we had previously designated the monoaromatic catabolic - mac - operon) the sequence of which has been made available by the present inventors for the first time. This is described in more detail below, and in Example 9. Thus an inducible promoter and/or operon proteins may be identified by providing a nucleic acid molecule having a nucleotide sequence identical to, complementary to, or specifically hybridisable with, the corresponding part of a known, appropriate, mycolic acid bacterial sequence, such as the sequence shown in Fig. 4. Preferably parts of the sequence are used as probes, preferably of at least 100 nucleotides (but shorter sequences may be employed under high stringency conditions). The use of primers based on the sequence to screen and identify target sequences by PCR is also envisaged.

The identified putative inducible promoter can then be tested to see if it is operational as described in more detail below. Briefly, the putative promoter is provided in a vector upstream of a protein coding sequence (e.g. a reporter gene) at a position in which it is believed to be operatively linked to that coding sequence. A suitable host is transformed with the resulting vector. The presence or absence of the coding sequence expression product, in the presence of the inducing molecule, is determined. For putative transport proteins or catabolic enzymes identified by homology, function can be confirmed as described below.

As an alternative, or in addition to, homology screening, operon proteins which have catabolic enzymic activity can be screened for by their activity. For instance by contacting substrates for the enzymes (the analytes) with the host cells, or extracts therefrom, and observing for degradation products.

This approach can be used when the enzyme concerned may be successfully expressed in the recombinant host cell. For example, the R. corallina ohp operon was isolated by screening recombinant E. coli for expression of a catechol 2,3-dioxygenase activity induced in R. corallina when grown on monoaromatic compounds such as toluene. The substrate of this enzyme is catechol, a water soluble 2 hydroxyphenol which does not lyse E. coli.

In fact, R. corallina does express a mac catechol 2,3-dioxygenase activity in the presence of toluene. However that activity was not isolated in E. coli. Instead, the ohp-associated catechol 2,3-dioxygenase activity was isolated. This enzyme is induced by orthohydroxyphenylpropionic acid in the medium, although it does cleave catechol. A likely reason for the isolation of the ohp enzyme (rather than the mac one) is that functional screening in E. coli, even in those cases where it is possible, will depend not only on the requisite activity being expressed by the host, but also on the relative efficiency with which it is expressed. Thus using E. coli as the host, and using a broadly specific enzyme screen, those genes from nocardioform actinomycetes which are most efficiently expressed will be preferentially isolated.

Additionally, other potential substrates/analytes e.g. toluene are highly toxic to E. coli and may cause its membrane to destabilise leading to cell lysis. Further, gene isolation by function is limited to those genes that are expressed in the test bacterium. Because of their evolutionary distance from the mycolic acid

bacteria, established cloning hosts such as E. coli or Gram-positive bacteria such as Bacillus subtilis and Staphylococcus aureus may not effectively recognise mycolic acid bacterial gene regulatory signals and/or may not transport or survive in the presence or xenobiotics per se. Therefore, isolation by acquisition of novel-phenotype cannot easily be accomplished in these hosts.

In addition, when screening for proteins involved in binding or transporting the analyte, or transducing this binding event to the inducible promoter (e.g. transcription factors), it may be necessary to use a host in which other elements of the entire system (i.e. promoter and/or signal gene or catabolic enzymes) are present in order to demonstrate activity.

In order to circumvent these problems, in a most preferred embodiment of the second aspect, vectors comprising the inducible promoter and/or operon proteins are identified by means of a functional screen in a second host. This can avoid the difficulties described above. Preferably this second host is a suitable mycolic acid bacterium.

In order that the vectors can be maintained in the mycolic acid bacteria, they must encode replicons which can function in mycolic acid bacteria. These replicons can be those known in the art (e.g. based on characterised mycolic acid bacterial plasmids pSR1 (Batt et al., 1985). Alternatively the present inventors have provided a novel method of generating supercoiled or circular plasmid DNA from mycolic bacteria, and this method forms one part of the present invention. The diversity of the mycolic acid bacteria means that it is unlikely that a single replicon will be sufficient to construct biosensors in all strains encountered. Novel replicons which can be used either alone or in conjunction (two or more per vector) with other replicons to expand host range therefore provide a useful contribution to the art.

Thus, using the supercoiled/plasmid method of DNA isolation detailed in Example 4, two previously uncharacterised plasmids pRC100 and pRC158 have been discovered in soil mycolic acid bacteria Rhodococcus corallina and mycolic acid bacterium strain RC158 respectively.

Strain RC158 contains a supercoiled plasmid of approximately 14.57 kb. The plasmid, designated pRC158, contains at least five EcoRI restriction enzyme sites which can be used to digest the plasmid into a specific restriction pattern of five major restriction fragments of 4.3, 3.3, 2.9, 2.2 and 1.6 Kb DNA respectively. An approximately 100 kb plasmid, pRC100, was isolated from R. corallina

Replicons may be identified from novel plasmids by screening fragments obtained therefrom in disabled vectors containing marker proteins (for instance based on pJP7 described below) to see if they can replicate in mycolic acid bacteria.

Novel plasmids isolated using the method, and novel replicon elements isolated from them, form a fourth aspect of the present invention. These, and existing replicons, may be used to construct cloning vectors which replicate in several mycolic acid bacterial strains. Thus it is possible to clone, isolate by function and express specific genes from not only a single "type strain" as is the common practice in molecular biology but also in a variety of mycolic acid bacteria.

It is preferable that the transfer of the vectors comprising the putative inducible promoters and/or operon proteins to the second host (preferably mycolic acid bacteria) from the first host (preferably an established cloning systems such as E. coli) be achieved using bacterial conjugation. Experiments have shown that restriction enzyme activity in newly isolated mycolic acid bacteria effectively limits the efficiency of electroporation of incorrectly methylated plasmid DNA to

very low, or undetectable levels. It is known that most restriction enzymes preferentially act on double stranded DNA substrates. It is known that conjugative DNA transfer, however, involves a single-stranded DNA intermediate and is thus relatively immune to restriction. It is known that the IncPa conjugative plasmid RP4 can transfer its DNA into a wide range of bacteria by conjugation. Accordingly, a series of conjugatively mobilizable mycolic acid bacteria / E. coli shuttle vectors have been constructed by incorporation of a 440 bp region of the RP4 plasmid encoding the origin of transfer (pJP8 figure 1). Experiments have shown that RP4 oriT vectors can be complemented in trans for tra functions allowing conjugative mobilization into a variety of mycolic acid bacteria at high efficiency.

The vectors for use in the most preferred embodiment of second aspect of the invention (i.e. functional screening in a second host), themselves form a fifth aspect of the present invention, such vectors typically comprising:

(a) a replicon for mycolic acid bacteria
(b) a replicon for E. coli
(c) a conjugative origin of transfer
(d) a lambda cos site

An example of such a vector is that termed pJP8 (Figure 5). This comprises (a) pCY104oriV, (b) pBR322 oriV (c) RP4 oriT , and (d)a cos site; however it will be apparent to those skilled in the art that any of these could be substituted for a sequence having similar function, for instance substituting pRC100 or pRC158 minimal replicon sequences for the novel pCY104 replicon.

Further plasmids are pRV1 and pJH6 which comprise oriV (for replication in E. coli); oriT (for transfer); Kan (antibiotic marker); pSR1 (for replication); a cos site.

In use such vectors will further comprise a fragment containing the putative inducible promoter and/or operon

proteins and optionally a signal protein, such as have been described above.

Thus a gene library can be constructed in a mobilizable cosmid shuttle vector such as pJP8. After in vitro packaging, cosmids can be recovered by adsorption to E. coli carrying mcrABC mrr hsdSRM recA recO. Given the size of the mycolic acid genome (approximately 4 Mb) a 99% confidence gene library requires approximately 2500 colonies.

To screen for specific functions (either a complete reaction pathway or specific reactions) the packaged cosmids may be adsorbed to E. coli mcrABC mrr hsdSRM recA recO containing an IncP plasmid such as RK2. Since the RK2 plasmid encodes several antibiotic resistance genes, it is modified by random mutagenesis to disable antibiotic resistance genes which are also used as markers in the cosmid vector. From this transformed strain, the mobilizable cosmid shuttle vector may be conjugated into a wide variety of mycolic acid bacteria for functional screening. In any such screen, the choice of mycolic acid bacterial strain will be governed by the known catabolic functions of the strain. Thus entire pathways may be isolated by screening for gain of function. Alternatively, if a particular strain is known to require only one or a few catabolic activities these may be screened for by complementation.

Another novel shuttle vector, pRV1, can be recovered with high efficiency in a suitable E.coli host, and then transfer to a mycolic acid bacterial strain via conjugation (which minimises host restriction difficulties) for screening. Thus, in this embodiment, the E coli strain is just an interim host. Optionally conjugative systems can be put into place in this interim host to directly allow mating to follow phage adsorption, thus minimising the period in E.coli.

By incorporation of a signal gene adjacent to the cloning site in pJP8 or pRV1 used to construct the gene

library, transconjugant mycolic acid bacteria can be screened for inducible expression of a signal protein such as luciferase in the presence of specific molecules. This will rapidly isolate environmentally responsive promoter/operator/regulator elements.

Once identified, by any of the methods of the second aspect of the invention above, the putative inducible promoter and/or operon proteins may be modified by subcloning mutagenesis (typically within E. coli) and screened for enhanced function in mycolic acid bacteria.

The term 'modified' is used to mean a sequence obtainable by introducing changes into the full-length or part-length sequence, for example substitutions, insertions, and/or deletions. This may be achieved by any appropriate technique, including restriction of the sequence with an endonuclease followed by the insertion of a selected base sequence (using linkers if required) and ligation. Also possible is PCR-mediated mutagenesis using mutant primers.

It may, for instance, be preferable to add in or remove restriction sites in order to facilitate further cloning.

Alternatively, it may be particularly desirable to modify the binding protein/agent in order to modify its specificity and/or affinity for analyte.

Modified sequences according to the present invention may have a sequence at least 70% identical to the sequence of the full or part-length inducible promoter or operon protein as appropriate. Typically there is 80% or more, 90% or more 95% or more or 98% or more identity between the modified sequence and the authentic sequence. There may be up to five, for example up to ten or up to twenty or more nucleotide deletions, insertions and/or substitutions made to the full-length or part length sequence provided functionality is not totally lost.

Modified promoters and/or operon proteins can be

screened for functionality as described above in relation to isolating novel elements.

Nucleic acid encoding the authentic or modified promoter and/or genes encoding the operon proteins (plus such modified proteins themselves) identified or obtained by the method of the second aspect of the invention form a sixth aspect of the invention.

Thus one embodiment of the sixth aspect is the R. corallina ohp locus described in Figures 3 and 4 including the promoter and individual operon proteins encoding therein, and modifications thereof.

The authentic or modified promoter identified or obtained by the method of the second aspect of the invention may be used to inducibly express a heterologous signal protein in a transformed host; this use forms a seventh aspect of the present invention.

In one embodiment of the seventh aspect, there is disclosed a method of transforming a host with a vector encoding the inducible promoter as described above, operably linked to the signal gene (e.g. encoding luciferase).

The vector used in the seventh aspect may remain discrete in the host. Alternatively it may integrate into the genome of the host.

For a potential host (e.g. Corynebacterium) which does not express or generate the other components of the system which may be required to give biosensor function (for instance the operon proteins such as the transport protein to transport analyte into the cell; binding protein to bind analyte thereby inducing the promoter activity; cofactors required for signal protein activity etc.) these components can be added exogenously in order to perform the methods of the first aspect, or can be encoded on the vector used to introduce the inducible promoter or supplied *in trans* on a separate nucleic acid. Indeed, as stated above, any transport and binding proteins required for the functionality of the inducible

promoter will frequently form part the operon containing the promoter, and may thus be identified and isolated alongside it using the methods disclosed above.

Preferably, however, the host (e.g. a mycolic acid bacterium, either the same or different to that which provided the source of the inducible promoter, but preferably the same) will itself naturally express the other components of the system required to give biosensor function. This ensures all the required gene products for biosensor function are present.

Indeed in this latter case, the signal protein gene may be introduced into the host such that it is operably linked to an existing inducible promoter. In this embodiment of the seventh aspect of the invention the identification and or isolation of the promoter or associated proteins as described above ultimately provides the information required to allow targeting of the gene into this region. Typically this will be achieved by initiating targeted integration using aspects of the sequence forming part of the promoter region or operon.

Direct integration of a signal gene system such as luciferase (e.g. luxAB operon) into an environmentally responsive regulon in a mycolic acid containing bacterium may be more efficient than approaches based on isolation of gene(s) and its/their characterisation followed by construction of the biosensor. This integration can be achieved by transposition or by illegitimate or legitimate recombination between a genetic construct introduced into the cell and the target operon or gene cluster located on either the chromosome or an episomal element. In situations where a gene cluster or operon has been identified as above, by either screening in E. coli or direct functional cloning in a mycolic acid bacterium, site-specific recombination may be used to direct integration of the signal gene(s) (such as luciferase) into the regulon.

Vectors for use in the seventh aspect of the invention, form an eighth aspect of the invention. Such vectors will typically include: (a) the signal gene, plus (b) the inducible promoter, operably linked to the signal gene, or a sequence capable of initiating recombination of the signal gene such that it becomes operably linked with the inducible promoter. Further operon proteins (optionally modified) may also be included in the vector.

Vectors of the eighth aspect of the invention can be readily constructed on the basis of the present disclosure, for instance based on pJP7 (Figure 6) which is described in more detail below.

Strain derivatives encoding different gene dosage levels of the promoter/signal gene can be created by integration of the construct into the chromosome (low copy number/low sensitivity) or by use of medium or high copy number plasmids (medium or high sensitivity).

A ninth aspect of the invention is a (biosensor) host transformed with the vectors of the eighth aspect.

In using the transformants of the ninth aspect in the methods of the first aspect, the signal (such as bacterial luciferase) may be detected extracellularly using a photomultiplier or photodiode or any other photosensitive device. This maintains the cell integrity and thus resistance to environmental shock.

Also embraced within the scope of the present invention are kits for performing the various aspects of the invention. For instance a kit suitable for use in the first aspect may comprise a preparation of the microorganism, plus further means for carrying out the contact or observation steps e.g. buffers, co-factors (e.g. luciferin for addition to luciferase). A kit for performing the second aspect may include any of the following: selective buffer, a non-ionic detergent, any means for carrying out the screening process (e.g. primers, probes, substrates for catabolic enzymes, vectors for transfer into a second host). Kits for

performing the seventh aspect may include vectors for generating biosensors plus other means for transforming hosts with them (e.g. buffers etc.).

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments falling within the scope of the present invention will occur to those skilled in the art in the light of these.

Figures

Figure 1 - shows an agarose gel on which digestions of the novel plasmid pRC100 has been run, as described in Example 5.

Figure 2 - shows an agarose gel on which digestions of the novel plasmid pRC158 has been run, as described in Example 5.

Figure 3 - shows a schematic view of the R. corallina ohp operon obtained by functional screening in E. coli, as described in Example 7. The schematic shows location of predicted genes: Regulator, Transport, Monooxygenase, Hydroxymuconic semialdehye hydrolase, Alcohol dehydrogenase. Initiator and terminator codons are shown as half height and full height lines respectively. Base coordinates refer to the Figure 4 sequence. The location of predicted promoter regions and direction are indicated by arrows. The molecular weights and coordinates of ohp genes are tabulated.

Figure 4 - shows the complete listing of the R. corallina ohp operon as described in Example 7. It includes a portion of a putative nitropropane promoter (5' of the regulator).

Figure 5 - shows a schematic diagram of the pJP8 vector of the present invention, as described in Example 8. Plasmid size is about 8.51 kb. pJP8 is a mycolic acid bacterium - E. coli mobilizable cosmid vector. It carries pCY104 replicon; is Kanamycin resistant 15 μg/ml mycolic acid bacteria, 50 μg/ml E. coli. It also carries lambda cos site, RP4 oriT site and a multiple cloning site.

Figure 6 - shows a schematic diagram of the pJP7 vector of the present invention, as described in Example 9. Plasmid size is about 10.66 kb. pJP7 is a mobilizable E. coli/Rhodococcus/Nocardia suicide/luciferase integration vector encoding luxAB signal genes, sacB gene

and thiostreppton resistance in Rhodococcus/Nocardia only up to 75 μg/ml (typically 1-10 μg/ml used in selections). The vector is RP4/RK2 mobilizable. By cloning a region of homology into the region upstream of the luxAB cassette, insertion can be targeted.

Figure 7 - shows a schematic diagram of the pRV1 vector of the present invention, as described in the Examples below. Plasmid pRV1 comprises a minimal pSR1 replicon (Archer & Sinskey, 1993 J Gen Microbiol 139: 1753-1759) which allows replication in C glutamicum. The pUC replication origin (Yanish et al, 1985 Gene 33: 103-119) allows replication in E. coli. Also included are a kanamycin resistance marker and the RP4 origin of conjugative transfer oriT. Transcription counterclockwise in the insert is terminated by the E. coli trpA terminator. Transcription clockwise into the insert may be initiated by the E. coli lac UV5 promoter.

Figure 8 - shows a schematic diagram of the pJH6 vector of the present invention, as described in the Examples below. This encodes the pSR1 replicon (*supra*) and the pBR322 replicon for replication in E. coli. Antiobiotic resistance markers are ampicillin (E. coli) and kanamycin (E. coli and mycolic acid bacteria). Transcription across the insert can be provided by exogenous expression of the T7 RNA polymerase (in vitro or in vivo).

Examples

Example 1 - A novel medium for oligotrophic screening

"MMRN" is prepared as a multicomponent stock to avoid the production of uncharacterised compounds during autoclaving. A "basic salts" stock is prepared containing 6g/L Na_2HPO_4; 3g/L KH_2PO_4; 1g/L NaCl; 4g/L $(NH_4)_2SO_4$; adjusted to pH 7.4 and made up to 989 mls with distilled water and autoclaved. A "100x A salts"

solution is prepared consisting of 20g/L $MgSO_4$; 2000 mg/L $FeSO_4.7H_2O$; 200 mg/L $FeCl_3$; 200 mg/L $MnSO_4.H_2O$ is prepared in distilled water and autoclaved. A "1000x B salts" solution consisting of 500 mg/L $ZnSO_4.7H_2O$; 200 mg/L $CuCl_2.2H_2O$; 200 mg/L $Na_2B_4O_7.10H_2O$; 100 mg/L $(NH_4)_7Mo_6O_{24}.4H_2O$ is prepared in distilled water and autoclaved. To prepare 1 litre of MMRN, sterile solutions of 989 mls basic salts, 10 mls 100 x A salts, 1 ml 1000 x B salts are combined. For solid media, agar is added to 1.4% w/v. Carbon-energy sources are supplemented to 80 μM final concentrations for soluble molecules, or as vapour for insoluble molecules (where their concentration is decided by their individual partition coefficients generally ranging from 3 to 40 μM). Petri plates or liquid cultures are incubated at 28°C to 30°C for up to 72 hours to accumulate sufficient biomass for genetic and biochemical testing.

Example 2 - Isolation of novel strains of mycolic acid containing bacteria from environmental samples using an oligotrophic screen and MMRN

Novel strains are a source of genetic diversity from which biosensors specific for particular xenobiotic compounds can be constructed. To isolate mycolic acid bacteria, for example Rhodococcus / Nocardia, from an environmental matrix such as soil, a rapid isolation technique is required. Isolation of bacteria from soil using standard laboratory media containing eutrophic levels of carbon preselects for eutrophic bacteria which can grow rapidly under these conditions. Oligotrophic bacteria such as Rhodococcus / Nocardia are rarely successfully isolated on such rich media. This can be carried out using MMRN to specifically enrich for and subsequently purify strains of mycolic acid-containing

bacteria which encode catabolic pathways whose expression is induced by a given xenobiotic. This methodology identifies molecules which are not only substrates, but are necessary and sufficient to induce the appropriate catabolic pathway. Soil suspensions from a matrix likely to express a desired phenotype (for instance a site known or believed to have been contaminated with a particular xenobiotic) can be used to inoculate MMRN supplemented with an oligotrophic level of a easily utilised carbon source (50μM). This provides an initial oligotrophic screen. Oligotrophic mycolic acid-containing bacteria are slow growing and may be expected to have formed colonies after 72 hours incubation at 28°C on MMRN paraffin. The incubation temperature appears to be highly selective of soil Nocardioform bacteria; Petri plates incubated at temperatures above 30°C fail to show detectable colonies. Colonies growing on alkanes can be initially screened for Nocardioform phenotype, selecting for crumbling, crenellated colonies, (possibly mucoid on rich media). Gram- and Ziehl-Neelsen-staining tests rapidly identify Gram-positive, mycolic acid-containing bacteria (Place a slide carrying a heat fixed film on a slide carrier over a sink. Flood with carbol fuchsin solution (basic fuchsin 5g; phenol, crystalline, 25g; 95% or absolute ethanol 50 ml; distilled water 500 ml) and heat until steam rises. Leave for 5 minutes, heating occasionally to keep the stain steaming. Wash with distilled water. Flood slide with 20% v/v sulphuric acid; wash off with distilled water, and repeat several times until the film is a faint pink. Finally wash with water. Treat with 95% v/v ethanol for 2 minutes. Wash with distilled water. Counterstain with 0.2% w/v malachite green. Wash and blot dry. Acid and alcohol fast organisms are red, other organisms are green).

Mycolic acid-containing bacteria may then be screened for specific hydrocarbon-inducible catabolic pathways using MMRN supplemented with the target

xenobiotic pollutant. Strains for which the target molecule is growth inducing may then be isolated and used to as a source of genetic regulatory elements for biosensors or as specific biocatalytic functions. Using this protocol mycolic acid containing bacteria have been and may be rapidly identified with novel and useful catabolic properties. This approach is also useful for identification and isolation of mycolic acid containing bacteria with biocatalytic properties.

Example 3 - Method for isolation of total DNA from mycolic acid bacteria

Bacterial strains were inoculated into 10 mls of MMRN supplemented with 500μM glucose 2% w/v L-glycine and incubated at 28°C for 30 to 40 hours. This medium supports relatively rapid growth of mycolic acid bacteria cells. The L-glycine present is misincorporated into peptidoglycan cell wall substantially weakening its resistance to osmotic shock (Katsumata, et al., 1984). Growth on MMRN appears to enhance the uptake of L-glycine and its apparent misincorporation into the cell arabinogalactan. During this growth phase, mycolic acid bacteria produce extensive surfactants which cause the accumulated biomass to clump into pellicles and exhibit a strong surface tension effect. These pellicles, which are highly resistant to lysozyme, may be broken up and the concentration of biosurfactants substantially reduced by washing the cell pellet in several culture volumes of 10 mM Tris pH8.0; 0.1% Tween 80 and finally resuspended in 1ml of 10 mM Tris HCl pH8.0, containing 10 mg/ml lysozyme. The lysozyme reaction is incubated 60 to 100 minutes at 37°C depending on the strain involved. Lysis is achieved by addition of 2% final (w/v) sodium dodecyl sulphate at 60°C 40 minutes. The nucleic acids are selectively purified from the cellular debris by sequential phenol, phenol: chloroform :isoamyl alcohol

(50:48:2 v/v) extractions. Nucleic acids are concentrated by ethanol precipitation in 2 M ammonium acetate. The nucleic acid pellet recovered is washed with 70% ethanol and resuspended in 100µl 10 mM Tris.HCl pH8.0, 1mM EDTA. 2 µl of this sample may be digested using restriction enzymes.

Example 4 - Method to isolate supercoiled/circular plasmid DNA from mycolic acid bacteria

50 mls Rhodococcus was cultured to mid-logarithmic phase in MMRN supplemented with 2% w/v L-glycine, 2% w/v D-glucose.

The cell pellet was washed in 10 mM Tris pH8.0 and 0.1% Tween 80. Resuspend cell pellet in 7.6 ml 6.7% sucrose; 50 mM Tris.HCl; 1 mM EDTA. Add 2 ml 40 mg/ml lysozyme in 10 mM Tris.HCl 1 mM EDTA. Incubate 37°C 15 minutes. Add 970 µl 250 mM EDTA, 50 mM Tris.HCl pH 8.0. Continue incubation for a further 105 minutes 37 °C. Lyse cells by addition of 600 µl 20% SDS 50 mM Tris.HCl, 20 mM EDTA pH 8.0. Incubate 55°C 30 minutes. Shear lysate by vigorous vortexing 30 seconds. Denature DNA by addition of 560 µl freshly prepared 3 M NaOH followed by gently mixing 10 minutes room temperature. Neutralise by addition of 1 ml 2.0 M Tris.HCl pH 7.0 with gentle mixing 10 minutes. Add 2.1 ml 20% SDS 50 mM Tris.HCl, 1 mM EDTA. Mix gently. Add 4.2 ml ice cold 5 M NaCl. Incubate on ice overnight or for several hours at least. Clear the cellular debris by centrifugation at 48000 g 4°C 90 minutes. The supernatant contains the DNA. Decant the supernatant by addition of an equal volume of ice cold isopropanol. Incubate -20°C 30 minutes. Pellet nucleic acids 4°C, 10000g 20 minutes.

Example 5: Novel plasmids and replicons obtained by the method of Example 4

Two multicopy plasmid replicons were isolated using the method of Example 4; pRC158 from strain RC158 and pRC100 from R. corallina.

Both plasmids have been digested with restriction enzymes to produce characteristic restriction patterns (Figures 1 and 2).

Plasmid pRC100, an approximately 100kb supercoiled circular plasmid present in R. corallina was prepared as described in the text. The agarose gel was loaded in lane 1 with Lambda DNA HindIII size markers (23,130 bp; 9,416 bp, 6,557 bp, 4,361 bp, 2,322 bp, 2,027 bp, 564 bp); lanes 2 to 9 inclusive were loaded with pRC100 digested with BamHI (5'GGATCC3'), Bc1I (5'TGATCA3'), Bg1II (5'AGATCT3'), EcoRI (5'GAATTC3'), HindIII (5'AAGCTT3'), KpnI 5'(GGTACC3'), SacI (5'GAGCTC3'), SalI (5'GTCGAC3') restriction endonuclease reactions which were carried out under standard conditions; lane 10 contains undigested (presumable supercoiled) pRC100 DNA; lane 11 pWW110/40121, lane 12 pWW110/4011; lane 13 pWW15/3202; lane 14 pUC18 lane 15 blank. The DNA fragments have been resolved on a 0.8% Agarose Tris-Acetate-EDTA gel. Southern blotting analysis using Gram-negative mono and polyaromatic catechol 2,3-dioxygenases failed to detect significant sequence conservation.

Plasmid pRC158 is a supercoiled plasmid of approximately 14.57 kb. The plasmid was digested with the EcoRI (5'GAATTC3') restriction endonuclease under standard conditions. The DNA fragments have been resolved on a 0.8% Agarose Tris-Acetate-EDTA gel. This pattern is unique and characteristic to pRC158. The plasmid contains at least five EcoRI restriction enzyme sites which can be used to digest the plasmid into a specific restriction pattern of five major restriction fragments of 4.3, 3.3, 2.9, 2.2 and 1.6 Kb DNA respectively.

These plasmids are relatively small, exhibit a high plasmid copy number and are easily isolated from

Rhodococcus / Nocardia. Therefore, they possess several characteristics which are suitable for the construction of Rhodococcus / Nocardia cloning vectors.

The DNA sequence of the minimal replicon regions of these plasmids may be determined by screening fragments obtained therefrom in disabled vectors containing marker proteins (for instance based on pJP7 described below) to see if they can replicate in mycolic acid bacteria.

Further plasmids e.g. pCY101 have also been isolated and sequenced using the methods of the present invention. The replicon from this plasmid was used in pJP8.

Example 6: Hybridisation screening for novel promoters and/or operon proteins

The test sample (host cells) are contacted with a nucleic acid molecule probe (preferably around 100 nucleotides or more) based on Figure 4 under suitable hybridisation conditions, and any test DNA which hybridises thereto is identified. Such screening is initially carried out under low-stringency conditions, which comprise a temperature of about 37°C or less, a formamide concentration of less than about 50%, and a moderate to low salt (e.g. Standard Saline Citrate ('SSC') = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7) concentration. Alternatively, a temperature of about 50°C or less and a high salt (e.g. 'SSPE'= 0.180 mM sodium chloride; 9 mM disodium hydrogen phosphate; 9 mM sodium dihydrogen phosphate; 1 mM sodium EDTA; pH 7.4). Preferably the screening is carried out at about 37°C, a form amide concentration of about 20%, and a salt concentration of about 5 X SSC, or a temperature of about 50°C and a salt concentration of about 2 X SSPE. These conditions will allow the identification of sequences which have a substantial degree of similarity with the probe sequence, without requiring the perfect homology for the identification of a stable hybrid. The phrase

'substantial similarity' refers to sequences which share at least 50% overall sequence identity. Preferably, hybridisation conditions will be selected which allow the identification of sequences having at least 70% sequence identity with the probe, while discriminating against sequences which have a lower level of sequence identity with respect to the probe. After low stringency hybridisation has been used to identify several clones having a substantial degree of similarity with the probe sequence, this subset of clones is then subjected to high stringency hybridisation, so as to identify those clones having a particularly high level of homology with respect to the probe sequences. High stringency conditions comprise a temperature of about 42°C or less, a form amide concentration of less than about 20%, and a low salt (SSC) concentration. Alternatively they may comprise a temperature of about 65°C or less, and a low salt (SSPE) concentration. Preferred conditions for such screening comprise a temperature of about 42°C, a form amide concentration of about 20%, and a salt concentration of about 2 X SSC, or a temperature of about 65°C, and a salt concentration of about 0.2 SSPE.

Example 7 - Cloning aromatic degradative operon from Rhodococcus corallina by functional screening in E coli

To demonstrate the potential mycolic acid bacteria (e.g. Rhodococcus / Nocardia) have as biosensors and biocatalysts as well as to validate the novel genetic tools and approach to cloning of the present invention, a gene cluster or operon associated with aromatic degradation was cloned and isolated from Rhodococcus corallina. This gene cluster / operon appears to be a broad substrate range monoaromatic degradative pathway and has been designated monoaromatic catabolic (mac) gene cluster or operon. R. corallina was isolated from pristine soil in Canada and is an acknowledged

Rhodococcus type strain. This strain encodes a broad range of catabolic activities which include toluene, benzoate, phenol, cumine, cyamine. Genetic induction of the toluene degradative pathway in R. corallina occurs when toluene is supplied as vapour. This is a level of less than 200 ppm in water. Therefore, the sensitivity inherent in the biology of Rhodococcus is very close to those levels expected for biosensors in industrial use. Similar experiments using a naphthalene utilising Rhodococcus which is also supplied as a vapour

Biochemical assays of ring cleavage dioxygenase activities in crude enzyme extracts of R. corallina cells grown on MMRN supplemented with different growth-inducing xenobiotics indicated that the molecular specificity of ring cleavage dioxygenase induction is good. Toluene induced the meta pathway (although some ortho activity was observed) whereas benzoate and phenol exclusively induces the ortho pathway. Xylene, which is very closely related to toluene does not act as a growth inducing substrate. The closely related compounds toluene and benzoate but not xylene induce different ring-cleavage enzymes despite their relatively similar molecular shape. This behaviour and absence of induction with xylene suggests that the receptor for these or metabolites derived from these molecules is sensitive to minor electrostatic changes in their ligand. This strongly asserts that genetically constructed biosensors derived from these receptor molecules and their regulated promoter(s) will exhibit a level of specificity which exceeds that currently available as field test systems.

Since a clear catechol 2,3-dioxygenase activity was induced by toluene, but not by benzoate (indicating that the meta pathway in this strain is specifically induced by toluene), the catechol 2,3-dioxygenase activity can be used as a marker for gene(s), gene cluster(s) or operon(s) involved in its degradation.

The R. corallina catechol 2,3-dioxygenase structural

gene was isolated by functional screening of a partial Sau3A restriction enzyme digest-generated gene library in E. coli hsdRMmcrAB for using the commercially available cosmid cloning vector pWE15 (Wahl et al., 1987).

Because only a single enzyme activity has been used as a functional marker rather than complete acquisition of a phenotype and given the diversity of Rhodococcus / Nocardia metabolism and the genetic incompatibility between mycolic acid bacteria and E. coli it is possible that numerous catechol dioxygenases may exist but only some will be expressed successfully in E. coli. To facilitate expression of cloned DNA irrespective of the presence of an indigenous promoter element, a phage T7 promoter is located adjacent to the pWE15 unique BamHI restriction site into which the rhodococcal DNA was inserted. Phage T7 RNA polymerase (a single polypeptide) is supplied in trans from pGP1-2Sm. As a functional screen for 2,3-dioxygenase activity, catechol was sprayed onto nutrient agar plates supplemented with 15 μg/ml kanamycin, 50 μg/ml streptomycin, 0.1 mM isopropyl thiogalactoside (IPTG) incubated at 30°C to accumulate biomass. The expression of T7 polymerase is repressed by temperature sensitive phage lambda repressor which is itself expressed from an IPTG induced lacUV5 promoter. Thus incubation at 42°C leads to induction of T7 polymerase expression and so transcription of the pWE15 insert region from the T7 promoter (i.e. one direction of transcript alone).

Using the pGP1-2Sm T7 expression system, two colonies were isolated which encoded the characteristics catechol 2,3-dioxygenase activity from R. corallina. From approximately 3000 colonies of individual primary clones of R. corallina gene library in an E. coli hsdRMmcrAB strain, two colonies were observed to produce a deep yellow colour indicative of catechol 2,3-dioxygenase activity (2-hydroxymuconic semialdehyde) when exogenous catechol was supplied in phosphate buffer

(0.1M pH7.4). These clones were designated clone #1 and clone #2. Restriction enzyme mapping of both clone #1 and clone #2 DNA showed that both encode overlapping regions of DNA but were otherwise nonsibling clones; this is compatible with a primary screening of a cosmid library.

Southern blot analysis of R. corallina total cellular and plasmid DNA confirmed that the isolated catechol 2,3-dioxygenase locus in clones #1 and #2 are contiguous with an approximately 35 kb region R. corallina genomic DNA. The common region to both clones is comprised of seven major EcoRI restriction fragments (8.3, 7.2, 5.2, 4.9, 4.3, 2.4, 2.3 Kb respectively 34.6 kb in total). To confirm the continuity and source of the clone #1 and clone#2 inserts, an aliquot of clone #2 DNA, which contained a slightly longer R. corallina DNA insert than clone #1, was used as a source of DNA to synthesise a radioactive probe to identify homologous DNA restriction fragments present in an EcoRI restriction digest of total cellular R. corallina DNA as well as other bacterial DNA samples. An randomly picked pWE15 clone which did not express catechol 2,3-dioxygenase was chosen as one control (cosmid clone "clone # 4") and E. coli genomic DNA were selected as control DNAs. At the level of accuracy of the gel, the coincidence of the catechol 2,3-dioxygenase clones #1 and clone #2 DNA inserts relative to the genomic R. corallina EcoRI and SmaI restriction maps indicated that no gross deletions or rearrangements had occurred during the cloning. Significantly, there was no evidence for a supercoiled plasmid location for the catechol 2,3-dioxygenase gene indicating that the locus is chromosomally encoded (although pRC100 has been isolated from R. corallina (see Figure 1) this strain does not encode large linear plasmids). To investigate the potential for gene homologs to be identified a Rhodococcus strain RC161 which was isolated from North East England and so is

distinct from R. corallina (which also degrades toluene via meta cleavage but was isolated form soil in Canada) was included in the Southern Blot. There were three RC161 EcoRI restriction fragments which exhibited significant DNA sequence conservation with R. corallina sequences in clone #2. The nature of these sequences is under investigation.

Colony hybridisation to the R. corallina gene library secondary screen using the 2.4 Kb EcoRI restriction fragment of clone #2 as a source of radioactive probe identified four cosmid clones, pWE15#C, pWE15#D, pWE15#B and pWE15#G encoding overlapping regions of the R. corallina chromosome. Thus a region of the R.corallina genome with a contiguous length of approximately 70 kb has been cloned and isolated. These cosmids will provide a source of R. corallina DNA for future experiments.

The 35 Kb region encoded by clones #1 and #2 was mapped using four six base recognition restriction enzymes. An analysis of the map does not indicate inverted DNA map elements which could be consistent with a transposable element. This does not, however, preclude this possibility existing.

The sequence of the operon is described in Example 9 below.

Further plasmids which may be used for screening in accordance with the methods of the present invention are as follows:

pRV1

This is shown in Figure 7. It encodes the pSR1 replicon for Corynebacterium, the pUC replicon for E.coli, the RP4 oriT and a minimal cos PCR product. The multiple cloning site is under the control of the lac operon promoter allowing expression in E. coli.

The cos sequence in currently available in cosmids

such as pWE15 (Stratagene) and is encoded within an approximately 1 Kb region. However experiments showed that cos induced structural instability in several different plasmids. Analysis of the cos region in lambda suggested that the instability may be due to high levels of transcription entering the plasmid cos site and or transcription through adjacent lambda coding sequences which flank cos in the standard cosmid cloning vectors. To avoid problems with these extraneous elements, using computer-aided sequence analysis, the present inventors designed oligonucleotide primers to amplify the minimal cos element, free from flanking genes which may induce instability and occupy valuable cloning space. Additionally, experiments indicated that the cos PCR product induced structural instability in vectors carrying it. Therefore the cos PCR product was cloned into pRV1 (a preferred shuttle vector of the present invention) into a transcriptional quiet region of the plasmid. Transcription was blocked using a transcriptional terminator (trpA terminator from E. coli). This construct combines cosmid function with a mycolic acid replicon, an E.coli replicon, a selectable marker, a conjugative oriT, and a unique BamHI cloning site.

Briefly, the plasmid was prepared by cleaving plasmid pWSTIB (Peoples et al, 1988 Mol Microbiol 2(1): 63-72) with NheI and SalI to clone the *C glutamicum* replicon into the mobilisable plasmid pK19mob (Shäfer et al, 1994 Gene 145: 69-73) to form a shuttle vector designated pJH4. The minimal Cos site from wild-type phage (Promega) was amplified by PCR using primers which introduced two XbaI sites (5′ TCTAGA 3′) into the fragment.

The primers were:

F: 127 5′ CGCTGATTTGTATTGTCTG 3′ 145

R: 502 5' GACTTCCATTGTTCATTCC 3' 484

The fragment was cloned into pJH4 to give pRV1.

pJH6

This is shown in Figure 8. It also encodes the pSR1 replicon for Corynebacterium, the pUC replicon for E.coli, the RP4 oriT and a minimal cos PCR product. Inserted genes are expressed under the T3 and T7 promoters which are controlled by temperature shift, allowing the controlled production of genes which may impose a lethal phenotype.

Briefly, the plasmid was prepared by cleaving plasmid pWE15 (stratagene) with Agl III enzyme to remove unwanted SV40 ori and Neo sties. The NheI/BstBI fragment of pK18mob (Shäfer et al, 1994 Gene 145: 69-73) was cloned into pWE15-small to add a kanamycin resistance marker known to work in *C glutamicum* and *E coli*. The plasmid pWSTI B (above) was cleaved with BgIII and BamH1 enzymes to clone the pSR1 origin of replication of *C glutamicum* into pWE15-small. Finally RP4(OriT) was amplified by PCR using the following primers, which incorporate AatII restriction site:

F: 51171 5' AAAAGACGTCGGTGCGAATAAGGGACAGTG 3' 51190
R: 51395 5' AAAAGACGTCACAAAACAGCAGGGAAGCAG 3' 51376

The amplified fragment was cloned into the AatII site of the pWE15-small-Km-pSR1 construct to form the shuttled vector designated pJH6.

<u>Example 8 - A method for gene isolation from mycolic acid-containing bacteria by functional screening in Corynebacterium glutamicum</u>

A key aspect of this invention is the ability to genetically manipulate a variety of strains or species of

mycolic acid-containing bacteria such as Rhodococcus / Nocardia in a simple, effective way so as to clone and isolate gene(s), gene cluster(s) or operon(s) with applications as biosensors or biocatalysis.

The closely related mycolic acid-containing bacterium Corynebacterium glutamicum may be used as a host to express Rhodococcus / Nocardia genetic material. C. glutamicum shares a common cell wall type and probably similar genetic regulation to Rhodococcus / Nocardia but since it has been used extensively for the industrial production of amino acids and nucleotides it has lost or may never had encoded significant xenobiotic catabolic activity. It therefore represents a good "naïve" host to express Rhodococcus / Nocardia genes.

Restriction enzyme activity in natural isolates of Rhodococcus / Nocardia effectively limits the efficiency of electroporation to very low, or undetectable levels. Most restriction enzymes recognise double stranded DNA exclusively. Because single-stranded DNA is a necessary product of a replication fork, normal restriction enzyme activity in bacterial cells is limited to double stranded DNA substrates. Conjugative DNA transfer in Gram-negative, and most probably between Gram-positive bacteria as well, involves a single-stranded DNA intermediate. Conjugative DNA transfer should thus, generally, be relatively immune to restriction.

pJP8

The pJP8 plasmid may be used to introduce the library in the first host into a suitable mycolic acid bacterium such as corynebacterium or any mycolic acid bacterium which does not encode the desired phenotype.

The pJP8 plasmid is shown in Figure 5. The shuttle vector carries a approximately 400 bp region of the IncP RK2 conjugative plasmid which encodes the origin of transfer. This may be complemented in trans by IncP tra functions maintained on a suitable compatible recombinant

plasmid, or as an integrated construct in the host chromosome or by RK2 itself (modified to disrupt its kanamycin resistance gene - a marker used for pJP8).

Conjugation involves "effective contact" between the donor and recipient cells, which in this case are E coli encoding complementing tra functions and bearing the mobilizable cosmid vector and a suitable mycolic acid bacterium respectively. Effective contact is the formation of a cytoplasmic bridge between the two cells through which conjugative DNA transfer occurs. Thus donor and recipient cells are grown to mid to late logarithmic phase of growth in Lauria Bertini broth and MMRN supplemented with suitable carbon source at 37°C and 30°C respectively. Donor and recipient cells are washed in prewarmed media and mixed on a solid support matrix such as Lauria Bertini Agar plate and incubated at 37°C for up to 16 hours. The mating mixture is scraped from the plate and resuspended in 30°C Lauria Bertini broth, from which serial dilutions are prepared and plated on MMRN agar supplemented with drugs to counter select against the donor and recipient and select for the transconjugant mycolic acid bacterium. Commonly, naladixic acid selects against the donor and kanamycin resistance selects against the recipient. Thus, on a plates supplemented with both only the transconjugant may grow. The plates are incubated at 30°C for 40 hours.

Example 9 - DNA sequence of the proximal region of R. corallina ohp locus

The DNA sequence of approximately 7 Kb of R. corallina chromosomal DNA surrounding a catechol 2,3-dioxygenase has been determined using automated dye terminator sequencing reactions. A schematic of the current state of the data is presented in Figure 3 which shows at least seven genes which have been identified by protein sequence conservation with known protein motif

data (nitropropane dioxygenase, a putative regulatory protein orfR, monoaromatic monooxygenase, hydroxymuconic semialdehyde hydrolase, catechol 2,3-dioxygenase, alcohol dehydrogenase).

The sequence of this region in shown in Figure 4.

The predicated gene organisation of the ohp associated region is indicative of the presence of possibly two different catabolic gene clusters or operons; one involving the nitropropane dioxygenase the other the ohp gene cluster or operon. Such a genetic organisation suggests that a set of divergent promoter elements are located between the predicted regulatory gene orfR and the ohp monooxygenase structural gene. Similarly, another promoter could map immediately upstream of the divergent open reading frame which has conservation to nitropropane dioxygenase.

Example 10 - use of the promoter obtained in Example 9

The R. corallina genes identified by sequence conservation or by function are listed in Figure 3. These are potentially useful as catalytic functions in various chemical transformations. The regulatory protein associated with the putative ohp operon (possibly encoded by orfR) is involved in the control of transcriptional initiation at its target promoter. This regulatory protein encodes the specificity of the operon and as such is likely to be central to the biosensor function. Subcloning of the regulatory protein and its target promoter could permit novel biosensor activities to be introduced into other Rhodococcus /Nocardia strains. In addition, if this regulatory protein is subjected to mutagenesis, mutants with altered function could be identified (using a luciferase promoter probe driven by the regulated promoter). The regulatory protein has a specific capability to bind its ligand from the environment. It is therefore potentially useful as a

protein adsorbent for specific molecules. This could have application in analytical chemistry sample preparation.

An analysis of the 5' region of the predicted genes and the catechol 2,3-dioxygenase reading frame has allowed us to predict the sequence involved in translational initiation. These "ribosome binding sites" can be used as sequence guides or templates for the creation of synthetic oligonucleotides encoding functional Rhodococcus / Nocardia translational initiation sites. Mutagenesis of this region can identify potentially up and down regulating base sequences changes.

The ohp promoter region which controls expression of the cloned operon lies between two putative genes (orfR regulatory gene and orfT transport gene). In addition to forming the basis of a biosensor, the promoter and its cognate regulatory system also could be used as an inducible expression system for Rhodococcus / Nocardia and other mycolic acid-containing bacteria. The sequence of this region encodes the binding sites and regulatory elements or operators involved in control of the ohp and possibly other closely linked genes or operons. This region constitutes the first defined sequence for a Rhodococcus / Nocardia promoter region. It can be used as a probe to identify similar sequences within other mycolic acid containing bacteria such as Rhodococcus / Nocardia. This promoter sequence could be used as a region of homology to drive targeted recombination / insertion of signal gene(s) such as Vibrio luciferase.

A vector such as pJP7 (Figure 6) may be used as follows:

The vector is a 'suicide vector' which can be used to drive expression of bacterial luciferase genes in R. corallina. A portion of the ohp promoter region (Figure 4) is ligated into the unique pJP7 XbaI restriction site downstream of an E. coli trpA transcriptional terminator.

The sacB gene allows counter selection for the integrated plasmid thus selecting for a second cross-over within the plasmid sequences to produce a gene replacement of the wild type gene with an interrupted gene including luciferase. An aspect to this technique is the ability to introduce DNA constructs into the target cell in a hyperrecombinogenic, non-replicating form. Conjugatively mobilised plasmids may represent just such a form in that they may be single-stranded form. Thus the conjugatively mobilised plasmid pJP7 which cannot replicate in mycolic acid bacteria could be used directly to integrate DNA constructs into a wide range of mycolic acid bacterial strains.

Example 11 - Biosensor

The biosensor of the present invention is typically a recombinant mycolic acid containing bacteria which may be Rhodococcus / Nocardia cell. The natural gene-regulatory system which activates expression of catabolic gene(s), gene cluster(s) or operon(s) in response to the presence of specific class or type of inducing naturally-occurring or xenobiotic carbon substrate(s) has been genetically manipulated to induce the expression of some signal gene(s), such as (but not limited to) the Vibrio or Photobacterium bacterial luciferase in the presence of the inducer. This manipulation may have involved either incorporation of the signal gene(s) into a chromosomally- or episomally-encoded regulon under the control of a suitable environmentally-regulated promoter, or by direct sub-cloning of the regulated promoter to a rhodococcal / nocardial plasmid or other replicon or episomal element encoding a promoter-less signal gene(s). The genetic manipulation effecting the substitution or supplementation of the natural genes with the signal gene(s)may involve integration of the signal gene(s) gene

cluster(s) or operon into the host chromosome, plasmid or other episomal element so as to place it under inducible regulatory control or subcloning of the analyte (particularly hydrocarbon)-responsive promoter to a multicopy plasmid. The integration may involve site-specific recombination, transposition or illegitimate or homology-driven DNA recombination which is another aspect of this invention; however other methods of DNA integration such as the use of polymerase chain reaction (PCR) are not ruled out.

Signal to noise ratio can be readily improved in the recombinant system by enhancing or optimising expression or function of the signal gene, which may be luciferase, by means of improved gene translational signals and/or increasing levels of transcription by either raising transcriptional rates, mRNA stability or gene dosage of the construct (by subcloning to a plasmid or iterative gene integrations into a chromosome, plasmid or other episomal element). Thus, for instance, transcriptional efficiency of the luciferase genes luxAB can be increased by substitution of the Vibrio translational initiation signals with those from the ohp operon.

References

von der Osten et al (1989) Biotechnol Letts 11: 11-16.
Wahl et al (1987) Proc Natl Acad Sci 84: 2160-2164.
Katsumata et al (1989) J Bacteriol 159: 306-311.

Claims

1. A method for identifying and/or isolating mycolic acid bacterial DNA encoding an inducible promoter which is induced in response to a specific analyte and/or associated operon proteins, the method comprising the steps of:
(a) culturing a source of mycolic acid bacteria in a selective medium containing said specific analyte and being selective for oligotrophic bacteria,
(b) identifying mycolic acid bacteria capable of subsisting on said medium,
(c) extracting DNA from said mycolic acid bacteria,
(d) incorporating said DNA into a vector,
(e) cloning said vector into a suitable host cell, and
(f) screening the host cell for said inducible promoter and/or proteins in order to identify vectors encoding it.

2. A method as claimed in claim 1 wherein the analyte is an environmental pollutant.

3. A method as claimed in claim 2 wherein the environmental pollutant is a hydrophic organic compound.

4. A method as claimed in any one of the preceding claims wherein the mycolic acid bacterium is a member of the Rhodococcus or Nocardia complex.

5. A method as claimed in any one of the preceding claims wherein the medium used in step (a) comprises less than <500 μM carbon supplement.

6. A method as claimed in any one of the preceding claims wherein the mycolic acid bacteria isolates are screened after or during step (b) to ensure an absence of catabolic repression.

7. A method as claimed in claim 6 wherein the catabolic repression screen is performed by assessing the concentration of an enzyme associated with the specific analyte of interest in (i) medium supplemented with the specific analyte, and (ii) medium supplemented with the specific analyte plus a high efficiency carbon source, and (iii) medium not containing the specific analyte but containing a high efficiency carbon source.

8. A method as claimed in any one of the preceding claims wherein the mycolic acid bacteria are grown on a medium comprising L-glycine prior to the DNA extraction at step (c).

9. A method as claimed in claim 8 wherein the mycolic acid bacteria are washed using 0.05 - 0.5 % (v/v) non-ionic detergent prior to the DNA extraction at step (c).

10. A method as claimed in any one of the preceding claims wherein the host cell of step (e) is an E coli strain carrying one or more of the mcrABC, mrr , hsdSRM recA or recO mutations.

11. A method as claimed in any one of the preceding claims wherein the host cell is screened for a sequence comprising an inducible promoter and/or operon proteins by using one or more oligonucleotide probes or primers corresponding to, or complementary to, a promoter and/or operon protein derived from a mycolic acid bacterium and selecting vectors which are complementary to, or specifically hybridisable with, said probe or primer.

12. A method as claimed in claim 11 wherein the oligonucleotide probe or primer comprises a sequence of at least 20, 30, 40, 50, or 100 nucleotides, said sequence corresponding to, or being complementary to, all or part of a contiguous sequence of the R. corallina ohp

operon.

13. A method as claimed in any one of claims 1 to 10 wherein the host cell is screened by:
(i) incorporating a sequence believed to comprise an inducible promoter plus optionally further operon proteins in a vector at a position in which it is operatively linked to a coding sequence,
(ii) transforming a host cell with said vector, and
(iii) determining the presence or absence of the coding sequence expression product in the presence of the analyte.

14. As method as claimed in any one of claims 1 to 10 wherein the host cell is screened for the inducible promoter and/or operon proteins by screening for an activity associated with the inducible promoter and/or operon proteins.

15. A method as claimed in claim 14 wherein the activity is an enzyme activity for which the analyte is a substrate.

16. A method as claimed in claim 15 wherein the enzyme activity is screened for by contacting the host cell or an extract thereof with a substrate for the enzyme and observing the cell or extract for enzymatically generated products of the substrate.

17. A method as claimed in any one of claims 14 to 16 wherein the vector in transferred from a first host cell of step (e) to a second host cell wherein the activity is screened.

18. A method as claimed in claim 17 wherein the second host is a mycolic acid bacterium.

19. A method as claimed in claim 18 wherein the second host is a Corynebacterium.

20. A method as claimed in any one of claims 17 to 19 wherein the vector in transferred from the first to the second host by bacterial conjugation.

21. A method as claimed in any one of claims 17 to 20 wherein the vector is shuttle vector capable of replication in the first and second hosts.

22. A method as claimed in claim 21 wherein the vector comprises two, three, four or five of the following elements: (i) a replicon for mycolic acid bacteria; (ii) a replicon for E. coli; (iii) a conjugative origin of transfer; (iv) a lambda cos site; (v) a sequence encoding an antibiotic marker gene.

23. A method as claimed in claim 22 wherein the elements are selected from a group comprising: pCY104oriV ; pBR322 oriV; RP4 oriT; pSR1.

24. A method as claimed in claim 23 wherein the plasmid is selected from: pJ8; pRV1; pJH6 as described herein.

25. A method of producing a modified inducible promoter and/or operon, the method comprising the step of modifying a nucleotide sequence encoding the inducible promoter and/or operon identified in accordance with the method of any of the preceding claims.

26. An isolated nucleic acid molecule comprising a nucleotide sequence encoding an inducible promoter and/or operon protein identified in accordance with the method of any one of claims 1 to 24 or produced by the method of claim 25.

27. A nucleic acid as claimed in claim 26 comprising a promoter region of the nucleotide sequence encoding the R. corallina ohp operon described in Figure 3.

28. A nucleic acid as claimed in claim 26 encoding one or more of the following proteins of the R. corallina ohp operon: Regulator REG; Transport TRANS; Monooxygenase MONO; Hydroxymuconic semialdehye hydrolase HMSH; Alcohol dehydrogenase ADH; and Catechol 2, 3-dioxygenase CDO.

29. A nucleic acid molecule comprising a sequence encoding a modified inducible promoter obtainable by the method claim 25 which is at least 70%; 80%; 90%; 95% or 98% identical to the sequence of the inducible promoter of claim 26 or claim 27.

30. A nucleic acid as claimed in any one of claims 26 to 29 further comprising a heterologous signal gene.

31. A nucleic acid comprising (a) a sequence capable of effecting site specific integration of a heterologous signal gene into the genome of host cell such that it is operably linked to an inducible promoter identified in accordance with the method of any one of claims 1 to 24; (b) a heterologous signal gene.

32. A vector comprising the nucleic acid of claim 30 or claim 31.

33. A vector as claimed in claim 32 comprising one or more of the following: luxAB signal genes; sacB gene; antibiotic resistance; RP4/RK2 mobilizing elements.

34. A vector as claimed in claim 33 which is pJP7 as described herein.

35. A method of transforming a host cell comprising use

of a vector as claimed in any one of claims 32 to 34.

36. A method as claimed in claim 35 wherein the host cell is transformed by site specific integration such that the signal gene is operably linked to an endogenous inducible promoter.

37. A method as claimed in claim 35 or claim 36 wherein the host cell is a mycolic acid bacterium of the same strain from which the inducible promoter and/or operon proteins were isolated.

38. A method of producing a biosensor comprising the method of any one of claims 35 to 37.

39. A biosensor host transformed with a vector as claimed in any one of claims 32 to 34 or as produced by the method claim 38.

40. A method of detecting the presence or absence of an analyte in a sample comprising the steps of:
(a) contacting the sample with a transformed microorganism which is a mycolic acid bacterium which expresses a binding agent capable of binding the analyte, wherein the binding of the agent to the analyte causes a detectable signal, and wherein said bacterium has been transformed such as to improve the detectability of the signal; and
(b) observing said bacterium for said detectable signal.

41. A method as claimed in claim 40 wherein the transformed microorganism is the biosensor of claim 39.

42. A method as claimed in claim 40 or claim 41 wherein the signal is detected by an increased expression of a heterologous signal protein from a signal gene.

43. A method as claimed in any one of claims 40 to 42 wherein the signal is detected photometrically.

44. A kit for performing the method of any one of claims 40 to 43 comprising (a) a biosensor as claimed in claim 39, plus (b) one or more further materials for performing the method.

45. A kit for performing the method of any one of claims 1 to 24 comprising two or more of the following (a) the selective buffer of claim 5; (b) a non-ionic detergent; (c) the primers or probes of claim 12; (c) the vector of any one of claims 21 to 24.

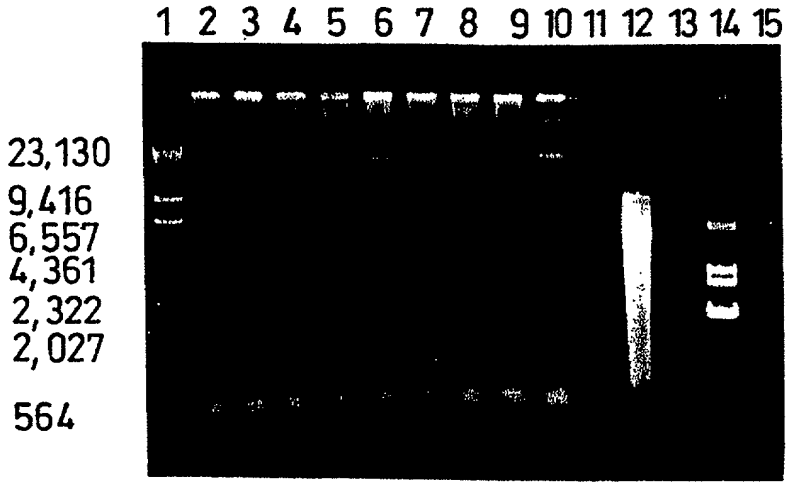

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
23,130
9,416
6,557
4,361
2,322
2,027
564

Fig. 1

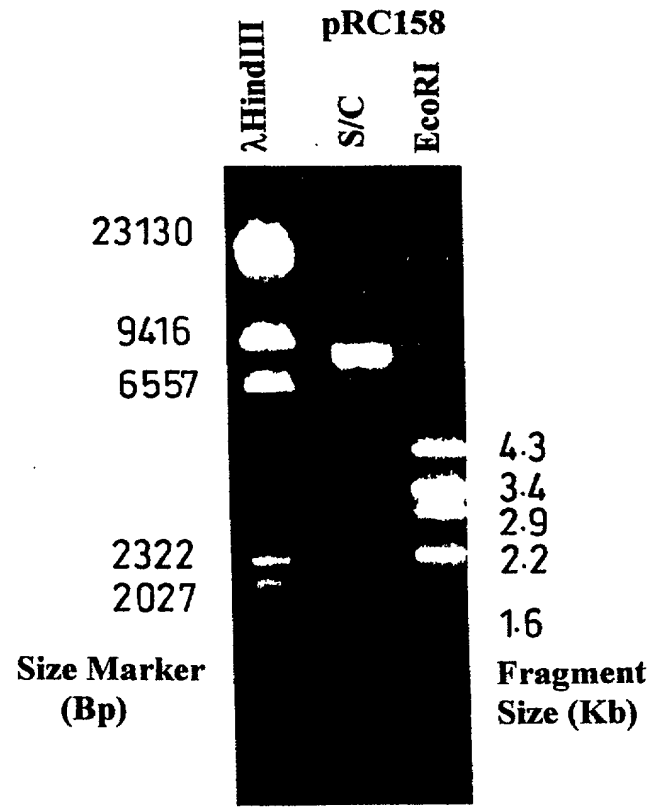
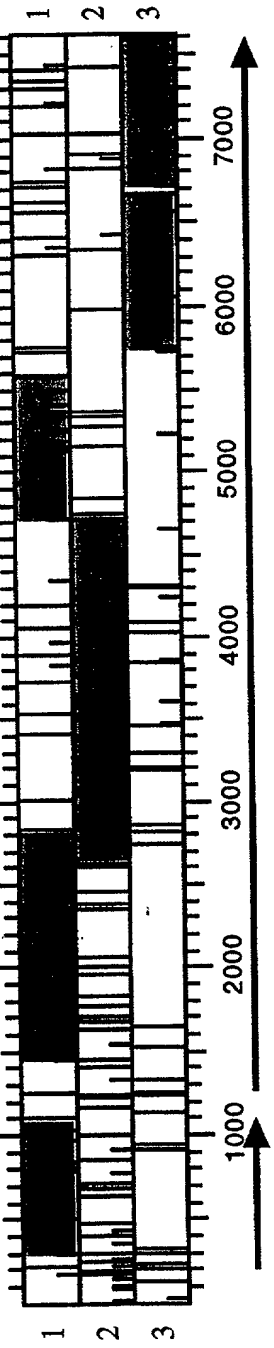

λHindIII
pRC158
S/C
EcoRI
23130
9416
6557
2322
2027
Size Marker (Bp)
4.3
3.4
2.9
2.2
1.6
Fragment Size (Kb)

Fig. 2

Fig 3



Gene	Initiator Codon	Terminator Codon	Molecular Weight
Regulator	295	1035	27102
Transport	1450	2805	47433
Monooxygenase	2810	4720	69650
Hydroxymuconic semialdehyde hydrolase	4717	5586	32770
Catechol 2,3-dioxygenase	5721	6665	33894
Alcohol dehydrogenase	6711	7580	30586

Fig 4

```
        10                  30                  50
         .         .         .         .         .         .
GAATTCCATGTTCTTCTCCTTGCATGTGGCCCGCGTTGCCGAGGGCACTGCTCGGCCTGT
CTTAAGGTACAAGAAGAGGAACGTACACCGGGCGCAACGGCTCCCGTGACGAGCCGGACA

        70                  90                  110
         .         .         .         .         .         .
CGCCCGCAGAGGGCGCATGTCCGGGTGCCTGGATATGGCGCGTACGGCGTGCCCTCCGGC
GCGGGCGTCTCCCGCGTACAGGCCCACGGACCTATACCGCGCATGCCGCACGGGAGGCCG

        130                 150                 170
         .         .         .         .         .         .
GTTAACCCCGAGGTTGGCCACGATGCCCCGGCCATCAGGTCTGGAATGCTAGCGTTCCAG
CAATTGGGGCTCCAACCGGTGCTACGGGGCCGGTAGTCCAGACCTTACGATCGCAAGGTC

        190                 210                 230
         .         .         .         .         .         .
ACGAAGGTAACCCACAGTGACTCACACCACAAGTACTAGAATGCAAGCTGTTGCGGTGAG
TGCTTCCATTGGGTGTCACTGAGTGTGGTGTTCATGATCTTACGTTCGACAACGCCACTC

        250                 270                 290
         .         .         .         .         .         .
CGCCGCGGCATAAGGGGGAGCCATGTCCGGGACGCCGACGGAAAGCCTGACTCGATGACC
GCGGCGCCGTATTCCCCCTCGGTACAGGCCCTGCGGCTGCCTTTCGGACTGAGCTACTGG
                                                      M   T

        310                 330                 350
         .         .         .         .         .         .
ACCACCGACACCGGCCCCAAGCCGGGCAGTGAGGCCGCCGCCCTGCTCGCCAATGTCCGC
TGGTGGCTGTGGCCGGGGTTCGGCCCGTCACTCCGGCGGCGGGACGAGCGGTTACAGGCG
T   T   D   T   G   P   K   P   G   S   E   A   A   A   L   L   A   N   V   R

        370                 390                 410
         .         .         .         .         .         .
ACCTCGGGGGCGCGGCTGTCCTCCGCGTTGTACGACATTCTGAAGAACCGGCTGCTCGAA
TGGAGCCCCCGCGCCGACAGGAGGCGCAACATGCTGTAAGACTTCTTGGCCGACGAGCTT
T   S   G   A   R   L   S   S   A   L   Y   D   I   L   K   N   R   L   L   E

        430                 450                 470
         .         .         .         .         .         .
GGGCGCTATGCGGCAGGCGAGAAGATCGTCGTCGAGTCGATCCGGCAAGAGTTCGGGGTG
CCCGCGATACGCCGTCCGCTCTTCTAGCAGCAGCTCAGCTAGGCCGTTCTCAAGCCCCAC
G   R   Y   A   A   G   E   K   I   V   V   E   S   I   R   Q   E   F   G   V

        490                 510                 530
         .         .         .         .         .         .
AGCAAGCAGCCCGTCATGGACGCTCTGCGCCGCCTGTCCAGCGACAAGCTGGTCCACATC
TCGTTCGTCGGGCAGTACCTGCGAGACGCGGCGGACAGGTCGCTGTTCGACCAGGTGTAG
S   K   Q   P   V   M   D   A   L   R   R   L   S   S   D   K   L   V   H   I

        550                 570                 590
         .         .         .         .         .         .
GTTCCCCAGGTCGGTTGCGAGGTCGTCTCCTACGCCCCGCGCGAAGTGGAAGACTTCTAC
CAAGGGGTCCAGCCAACGCTCCAGCAGAGGATGCGGGGCGCGCTTCACCTTCTGAAGATG
V   P   Q   V   G   C   E   V   V   S   Y   A   P   R   E   V   E   D   F   Y

        610                 630                 650
         .         .         .         .         .         .
ACCCTGTTCGGCGGTTTCGAAGGGACCATCGCCGCGGTAGCGGCCTCCCGGCGGACCGAG
```

```
TGGGACAAGCCGCCAAAGCTTCCCTGGTAGCGGCGCCATCGCCGGAGGGCCGCCTGGCTC
T  L  F  G  G  F  E  G  T  I  A  A  V  A  A  S  R  R  T  E

       670                 690                 710
        .                   .                   .
GCCCAGTTGCTGGAGCTGGACCTGATCTCGGCGCGGGTCGACGCCCTGATCACCTCCCAC
CGGGTCAACGACCTCGACCTGGACTAGAGCCGCGCCCAGCTGCGGGACTAGTGGAGGGTG
A  Q  L  L  E  L  D  L  I  S  A  R  V  D  A  L  I  T  S  H

       730                 750                 770
        .                   .                   .
GACCCGGTGGTCCGCGCCCGCGGGTACCGCGTGCACAACCGGGAGTTCCATGCGGCCATC
CTGGGCCACCAGGCGCGGGCGCCCATGGCGCACGTGTTGGCCCTCAAGGTACGCCGGTAG
D  P  V  V  R  A  R  G  Y  R  V  H  N  R  E  F  H  A  A  I

       790                 810                 830
        .                   .                   .
CACGCGATGGCGCACTCGCGGATCATGGAGGAGACCAGCCAGCGAATGTGGGATCTGTCG
GTGCGCTACCGCGTGAGCGCCTAGTACCTCCTCTGGTCGGTCGCTTACACCCTAGACAGC
H  A  M  A  H  S  R  I  M  E  E  T  S  Q  R  M  W  D  L  S

       850                 870                 890
        .                   .                   .
GACTTCTTGATCAACACCACCGGCATCACCAACCCGCTCTCGAGCGCACTGCCCGACCGG
CTGAAGAACTAGTTGTGGTGGCCGTAGTGGTTGGGCGAGAGCTCGCGTGACGGGCTGGCC
D  F  L  I  N  T  T  G  I  T  N  P  L  S  S  A  L  P  D  R

       910                 930                 950
        .                   .                   .
CAGCATGACCACCACGAAATCACCGAGGCCATCCGCAACCGTGACGCAGCTGCCGCCCGC
GTCGTACTGGTGGTGCTTTAGTGGCTCCGGTAGGCGTTGGCACTGCGTCGACGGCGGGCG
Q  H  D  H  H  E  I  T  E  A  I  R  N  R  D  A  A  A  A  R

       970                 990                 1010
        .                   .                   .
GAGGCCATGGAACGCCACATCGTCGGCACCATCGCAGTAATCCGCGACGAATCCAACGCC
CTCCGGTACCTTGCGGTGTAGCAGCCGTGGTAGCGTCATTAGGCGCTGCTTAGGTTGCGG
E  A  M  E  R  H  I  V  G  T  I  A  V  I  R  D  E  S  N  A

       1030                1050                1070
        .                   .                   .
CAGCTGCCGAGCTAGACCCCGATACCCGGGCCATCGACCGGCTCCGCTATCGCGCCACCT
GTCGACGGCTCGATCTGGGGCTATGGGCCCGGTAGCTGGCCGAGGCGATAGCGCGGTGGA
Q  L  P  S  *

       1090                1110                1130
        .                   .                   .
ACGCCGAGGGGGGACTCTCGGCCGTAGCGCTGCAGACGATCCACCGGCACCCTCCACGCT
TGCGGCTCCCCCCTGAGAGCCGGCATCGCGACGTCTGCTAGGTGGCCGTGGGAGGTGCGA

       1150                1170                1190
        .                   .                   .
GACCCCTGTCTCGCCCTAGAGGGCCGGCGCGCCGTCGATCACCTTTACCCTCATCCAGAG
CTGGGGACAGAGCGGGATCTCCCGGCCGCGCGGCAGCTAGTGGAAATGGGAGTAGGTCTC

       1210                1230                1250
        .                   .                   .
ACTTGCGTCACCCTCTATGCCCGAGTAGCGTCTGAACTAGACGTCTAGCATTCTAGTTGA
TGAACGCAGTGGGAGATACGGGCTCATCGCAGACTTGATCTGCAGATCGTAAGATCAACT

       1270                1290                1310
        .                   .                   .
GTGCTCCCTCTCGAAGATTCTCCAGAGAACCCCTCTCGAACATCCCCAGAAGAAAGGAGC
```

```
CACGAGGGAGAGCTTCTAAGAGGTCTCTTGGGGAGAGCTTGTAGGGGTCTTCTTTCCTCG

      1330                1350                1370
       .                   .                   .                   .
GGCCATGACGACCGCTTCGCACGCATCGTCCTTCGGGGCACGAGCCCACTTCCGCCCACA
CCGGTACTGCTGGCGAAGCGTGCGTAGCAGGAAGCCCCGTGCTCGGGTGAAGGCGGGTGT

      1390                1410                1430
       .                   .                   .                   .
GATCGGGGAAGCCCGACCGTGAGCACCACACCTACCTCCCCGACGAAGACCTCACCGCTG
CTAGCCCCTTCGGGCTGGCACTCGTGGTGTGGATGGAGGGGCTGCTTCTGGAGTGGCGAC

      1450                1470                1490
       .                   .                   .                   .
CGGGTAGCGATGGCCAGCTTCATCGGTACCACCGTCGAGTACTACGACTTCTTCATCTAC
GCCCATCGCTACCGGTCGAAGTAGCCATGGTGGCAGCTCATGATGCTGAAGAAGTAGATG
        M   A   S   F   I   G   T   T   V   E   Y   Y   D   F   F   I   Y

      1510                1530                1550
       .                   .                   .                   .
GGCACCGCGGCCGCGCTGGTATTCCCTGAGTTGTTCTTCCCGGATGTCTCGTCCGCGATC
CCGTGGCGCCGGCGCGACCATAAGGGACTCAACAAGAAGGGCCTACAGAGCAGGCGCTAG
G   T   A   A   A   L   V   F   P   E   L   F   F   P   D   V   S   S   A   I

      1570                1590                1610
       .                   .                   .                   .
GGAATCCTGTTGTCGTTCGCGACCTTCAGCGTTGGGTTCCTCGCCCGCCCGCTGGGTGGC
CCTTAGGACAACAGCAAGCGCTGGAAGTCGCAACCCAAGGAGCGGGCGGGCGACCCACCG
G   I   L   L   S   F   A   T   F   S   V   G   F   L   A   R   P   L   G   G

      1630                1650                1670
       .                   .                   .                   .
ATAGTGTTCGGGCACTTCGGTGACCGGGTCGGCCGCAAGCAGATGCTGGTGATCTCCCTG
TATCACAAGCCCGTGAAGCCACTGGCCCAGCCGGCGTTCGTCTACGACCACTAGAGGGAC
I   V   F   G   H   F   G   D   R   V   G   R   K   Q   M   L   V   I   S   L

      1690                1710                1730
       .                   .                   .                   .
GTCGGAATGGGCTCGGCCACCGTACTGATGGGATTGTTGCCCGGTTACGCCCAAATCGGG
CAGCCTTACCCGAGCCGGTGGCATGACTACCCTAACAACGGGCCAATGCGGGTTTAGCCC
V   G   M   G   S   A   T   V   L   M   G   L   L   P   G   Y   A   Q   I   G

      1750                1770                1790
       .                   .                   .                   .
ATCGCCGCCCCCATCCTGCTGACCCTGCTGCGCCTGGTGCAGGGCTTTGCCGTCGGCGGC
TAGCGGCGGGGGGTAGGACGACTGGGACGACGCGGACCACGTCCCGAAACGGCAGCCGCCG
I   A   A   P   I   L   L   T   L   L   R   L   V   Q   G   F   A   V   G   G

      1810                1830                1850
       .                   .                   .                   .
GAGTGGGGTGGAGCCACCCTGATGGCCGTCGAGCACGCCCCCACCGCGAAGAAGGGCTTT
CTCACCCCACCTCGGTGGGACTACCGGCAGCTCGTGCGGGGGTGGCGCTTCTTCCCGAAA
E   W   G   G   A   T   L   M   A   V   E   H   A   P   T   A   K   K   G   F

      1870                1890                1910
       .                   .                   .                   .
TTCGGATCCTTCTCCCAGATGGGGGCACCCGCCGGGACCAGCGTCGCAACCCTGGCGTTC
AAGCCTAGGAAGAGGGTCTACCCCCGTGGGCGGCCCTGGTCGCAGCGTTGGGACCGCAAG
F   G   S   F   S   Q   M   G   A   P   A   G   T   S   V   A   T   L   A   F

      1930                1950                1970
       .                   .                   .                   .
TTCGCGGTCTCCCAATTGCCCGACGAGCAGTTCCTGAGTTGGGGCTGGCGACTGCCGTTC
```

```
AAGCGCCAGAGGGTTAACGGGCTGCTCGTCAAGGACTCAACCCCGACCGCTGACGGCAAG
F  A  V  S  Q  L  P  D  E  Q  F  L  S  W  G  W  R  L  P  F

      1990                2010                2030
       .                   .                   .                   .
CTGTTCAGCGCGGTGCTGATCGTGATCGGGCTGTTCATTCGCCTGTCCCTGGCCGAAAGC
GACAAGTCGCGCCACGACTAGCACTAGCCCGACAAGTAAGCGGACAGGGACCGGCTTTCG
L  F  S  A  V  L  I  V  I  G  L  F  I  R  L  S  L  A  E  S

      2050                2070                2090
       .                   .                   .                   .
CCCGACTTCGCCGAGGTGAAGGCACAGAGCGCCGTGGTGCGAATGCCGATCGCCGAAGCG
GGGCTGAAGCGGCTCCACTTCCGTGTCTCGCGGCACCACGCTTACGGCTAGCGGCTTCGC
P  D  F  A  E  V  K  A  Q  S  A  V  V  R  M  P  I  A  E  A

      2110                2130                2150
       .                   .                   .                   .
TTCCGCAAGCACTGGAAGGAAATTCTCCTCATCGCGGGCACCTACCTGTCCCAAGGAGTG
AAGGCGTTCGTGACCTTCCTTTAAGAGGAGTAGCGCCCGTGGATGGACAGGGTTCCTCAC
F  R  K  H  W  K  E  I  L  L  I  A  G  T  Y  L  S  Q  G  V

      2170                2190                2210
       .                   .                   .                   .
TTCGCCTATATCTGCATGGCCTACCTCGTCTCCTACGGCACCACCGTCGCGGGGATCAGC
AAGCGGATATAGACGTACCGGATGGAGCAGAGGATGCCGTGGTGGCAGCGCCCCTAGTCG
F  A  Y  I  C  M  A  Y  L  V  S  Y  G  T  T  V  A  G  I  S

      2230                2250                2270
       .                   .                   .                   .
CGCACCTTCGCCCTGGCCGGAGTATTCGTCGCCGGCATCGTCGCCGTCCTCCTCTACCTC
GCGTGGAAGCGGGACCGGCCTCATAAGCAGCGGCCGTAGCAGCGGCAGGAGGAGATGGAG
R  T  F  A  L  A  G  V  F  V  A  G  I  V  A  V  L  L  Y  L

      2290                2310                2330
       .                   .                   .                   .
GTGTTCGGCGCTCTGTCCGACACTTTCGGCCGCAAGACCATGTACCTGCTCGGCGCCGCC
CACAAGCCGCGAGACAGGCTGTGAAAGCCGGCGTTCTGGTACATGGACGAGCCGCGGCGG
V  F  G  A  L  S  D  T  F  G  R  K  T  M  Y  L  L  G  A  A

      2350                2370                2390
       .                   .                   .                   .
GCGATGGGTGTGGTGATCGCCCCCGCCTTCGCACTGATCAACACCGGCAACCCGTGGCTG
CGCTACCCACACCACTAGCGGGGGCGGAAGCGTGACTAGTTGTGGCCGTTGGGCACCGAC
A  M  G  V  V  I  A  P  A  F  A  L  I  N  T  G  N  P  W  L

      2410                2430                2450
       .                   .                   .                   .
TTCATGGCCGCGCAGGTGCTGGTCTTCGGAATTGCAATGGCCCCCGCCGCCGGCGTGACA
AAGTACCGGCGCGTCCACGACCAGAAGCCTTAACGTTACCGGGGGCGGCGGCCGCACTGT
F  M  A  A  Q  V  L  V  F  G  I  A  M  A  P  A  A  G  V  T

      2470                2490                2510
       .                   .                   .                   .
GGCTCCCTGTTCACGATGGTCTTCGACGCGGACGTGCGCTACAGCGGTGTCTCTATCGGC
CCGAGGGACAAGTGCTACCAGAAGCTGCGCCTGCACGCGATGTCGCCACAGAGATAGCCG
G  S  L  F  T  M  V  F  D  A  D  V  R  Y  S  G  V  S  I  G

      2530                2550                2570
       .                   .                   .                   .
TACACCATCTCCCAGGTCGCCGGCTCCGCGTTCGCCCCGACGATCGCGACCGCCTTGTAC
ATGTGGTAGAGGGTCCAGCGGCCGAGGCGCAAGCGGGGCTGCTAGCGCTGGCGGAACATG
Y  T  I  S  Q  V  A  G  S  A  F  A  P  T  I  A  T  A  L  Y
```

```
      2590                2610                2630
       .                   .                   .                   .
GCCTCCACCAACACCAGCAACTCGATCGTGACCTACCTGCTGATCGTCTCGGCCATCTCG
CGGAGGTGGTTGTGGTCGTTGAGCTAGCACTGGATGGACGACTAGCAGAGCCGGTAGAGC
A  S  T  N  T  S  N  S  I  V  T  Y  L  L  I  V  S  A  I  S

      2650                2670                2690
       .                   .                   .                   .
ATCGTCTCGGTGATCCTGCTGCCCGGCGGCTGGGGGCGCAAGGGCGCTGCGAGCCAGCTC
TAGCAGAGCCACTAGGACGACGGGCCGCCGACCCCCGCGTTCCCGCGACGCTCGGTCGAG
I  V  S  V  I  L  L  P  G  G  W  G  R  K  G  A  A  S  Q  L

      2710                2730                2750
       .                   .                   .                   .
ACTCGCGACCAGGCCACCTCCACACCGAAAATGCCTGACACCGAAACATTTTCGACTCGG
TGAGCGCTGGTCCGGTGGAGGTGTGGCTTTTACGGACTGTGGCTTTGTAAAAGCTGAGCC
T  R  D  Q  A  T  S  T  P  K  M  P  D  T  E  T  F  S  T  R

      2770                2790                2810
       .                   .                   .                   .
ACAGTTCCGGACACCGCAGCATCCCTGCGCGTCCTCGACAAGTGAAGTGATGACAGACAT
TGTCAAGGCCTGTGGCGTCGTAGGGACGCGCAGGAGCTGTTCACTTCACTACTGTCTGTA
T  V  P  D  T  A  A  S  L  R  V  L  D  K  *     M  T  D  M

      2830                2850                2870
       .                   .                   .                   .
GAGTGACCACGACCGCACCTCCTACGACACCGACGTCGTGATCGTCGGCCTCGGCCCCGC
CTCACTGGTGCTGGCGTGGAGGATGCTGTGGCTGCAGCACTAGCAGCCGGAGCCGGGGCG
 S  D  H  D  R  T  S  Y  D  T  D  V  V  I  V  G  L  G  P  A

      2890                2910                2930
       .                   .                   .                   .
CGGTGGCACAGCGGCGCTTGCCCTGGCCAGCTACGGCATCCGCGTTCACGCCGTCTCGAT
GCCACCGTGTCGCCGCGAACGGGACCGGTCGATGCCGTAGGCGCAAGTGCGGCAGAGCTA
 G  G  T  A  A  L  A  L  A  S  Y  G  I  R  V  H  A  V  S  M

      2950                2970                2990
       .                   .                   .                   .
GTTCCCCTGGGTGGCGAACTCGCCGCGCGCGCACATCACCAACCAGCGCGCCGTCGAAGT
CAAGGGGACCCACCGCTTGAGCGGCGCGCGCGTGTAGTGGTTGGTCGCGCGGCAGCTTCA
 F  P  W  V  A  N  S  P  R  A  H  I  T  N  Q  R  A  V  E  V

      3010                3030                3050
       .                   .                   .                   .
GCTGCGTGACCTGGGCGTCGAAGACGAGGCGCGCAACTACGCCACCCCGTGGGACCAGAT
CGACGCACTGGACCCGCAGCTTCTGCTCCGCGCGTTGATGCGGTGGGGCACCCTGGTCTA
 L  R  D  L  G  V  E  D  E  A  R  N  Y  A  T  P  W  D  Q  M

      3070                3090                3110
       .                   .                   .                   .
GGGCGACACGCTGTTCACCACGAGCCTGGCCGGCGAGGAGATCGTCCGGATGCAGACCTG
CCCGCTGTGCGACAAGTGGTGCTCGGACCGGCCGCTCCTCTAGCAGGCCTACGTCTGGAC
 G  D  T  L  F  T  T  S  L  A  G  E  E  I  V  R  M  Q  T  W

      3130                3150                3170
       .                   .                   .                   .
GGGTACGGGCGATATCCGCTACGGGGACTACCTGTCCGGAAGCCCCTGCACGATGCTCGA
CCCATGCCCGCTATAGGCGATGCCCCTGATGGACAGGCCTTCGGGGACGTGCTACGAGCT
 G  T  G  D  I  R  Y  G  D  Y  L  S  G  S  P  C  T  M  L  D

      3190                3210                3230
       .                   .                   .                   .
CATTCCGCAGCCCCTGATGGAGCCGGTGCTGATCAAGAACGCCGCCGAACGTGGTGCGGT
```

```
GTAAGGCGTCGGGGACTACCTCGGCCACGACTAGTTCTTGCGGCGGCTTGCACCACGCCA
 I  P  Q  P  L  M  E  P  V  L  I  K  N  A  A  E  R  G  A  V

      3250                3270                3290
       .                   .                   .
CATCAGCTTCAACACCGAATACCTCGACCACGCCCAGGACGAGGACGGGGTGACCGTCCG
GTAGTCGAAGTTGTGGCTTATGGAGCTGGTGCGGGTCCTGCTCCTGCCCCACTGGCAGGC
 I  S  F  N  T  E  Y  L  D  H  A  Q  D  E  D  G  V  T  V  R

      3310                3330                3350
       .                   .                   .
GTTCCGCGACGTCCGCTCGGGCACCGTGTTCACCCAGCGAGCCCGCTTCCTGCTCGGTTT
CAAGGCGCTGCAGGCGAGCCCGTGGCACAAGTGGGTCGCTCGGGCGAAGGACGAGCCAAA
 F  R  D  V  R  S  G  T  V  F  T  Q  R  A  R  F  L  L  G  F

      3370                3390                3410
       .                   .                   .
CGACGGCGCACGATCGAAGATCGCCGAACAGATCGGGCTTCCGTTCGAAGGTGAACTCGC
GCTGCCGCGTGCTAGCTTCTAGCGGCTTGTCTAGCCCGAAGGCAAGCTTCCACTTGAGCG
 D  G  A  R  S  K  I  A  E  Q  I  G  L  P  F  E  G  E  L  A

      3430                3450                3470
       .                   .                   .
CCGCGCCGGTACCGCGTACATCCTGTTCAACGCGGACCTGAGCAAATATGTCGCTCATCG
GGCGCGGCCATGGCGCATGTAGGACAAGTTGCGCCTGGACTCGTTTATACAGCGAGTAGC
 R  A  G  T  A  Y  I  L  F  N  A  D  L  S  K  Y  V  A  H  R

      3490                3510                3530
       .                   .                   .
GCCGAGCATCTTGCACTGGATCGTCAACTCGAAGGCCGGTTTCGGTGAGATCGGCATGGG
CGGCTCGTAGAACGTGACCTAGCAGTTGAGCTTCCGGCCAAAGCCACTCTAGCCGTACCC
 P  S  I  L  H  W  I  V  N  S  K  A  G  F  G  E  I  G  M  G

      3550                3570                3590
       .                   .                   .
TCTGCTGCGCGCGATCCGACCGTGGGACCAGTGGATCGCCGGCTGGGGCTTCGACATGGC
AGACGACGCGCGCTAGGCTGGCACCCTGGTCACCTAGCGGCCGACCCCGAAGCTGTACCG
 L  L  R  A  I  R  P  W  D  Q  W  I  A  G  W  G  F  D  M  A

      3610                3630                3650
       .                   .                   .
GAACGGCGAGCCGGATGTCTCCGACGACGTTGTCCTCGAACAGATCCGGACCCTCGTCGG
CTTGCCGCTCGGCCTACAGAGGCTGCTGCAACAGGAGCTTGTCTAGGCCTGGGAGCAGCC
 N  G  E  P  D  V  S  D  D  V  V  L  E  Q  I  R  T  L  V  G

      3670                3690                3710
       .                   .                   .
CGACCCGCACCTGGACGTCGAGATCGTGTCGAGGTCCTTCTGGTACGTCAACCGGCAGTG
GCTGGGCGTGGACCTGCAGCTCTAGCACAGCTCCAGGAAGACCATGCAGTTGGCCGTCAC
 D  P  H  L  D  V  E  I  V  S  R  S  F  W  Y  V  N  R  Q  W

      3730                3750                3770
       .                   .                   .
GGCTGAGCACTACCAGTCCGGTCGAGTGTTCTGCGGCGGCGACGCGGTGCACCGGCATCC
CCGACTCGTGATGGTCAGGCCAGCTCACAAGACGCCGCCGCTGCGCCACGTGGCCGTAGG
 A  E  H  Y  Q  S  G  R  V  F  C  G  G  D  A  V  H  R  H  P

      3790                3810                3830
       .                   .                   .
GCCGAGCAGCGGGCTGGGCTCGAACACGTCCATGCAGGACGCGTTCAACCTGGCATGGAA
CGGCTCGTCGCCCGACCCGAGCTTGTGCAGGTACGTCCTGCGCAAGTTGGACCGTACCTT
 P  S  S  G  L  G  S  N  T  S  M  Q  D  A  F  N  L  A  W  K
```

```
      3850                3870                3890
       .                   .                   .
GATCGCGTTCGTCGTGAAGGGGTATGCAGGACCGGGTCTGCTCGAGTCCTACTCTCCTGA
CTAGCGCAAGCAGCACTTCCCCATACGTCCTGGCCCAGACGAGCTCAGGATGAGAGGACT
 I  A  F  V  V  K  G  Y  A  G  P  G  L  L  E  S  Y  S  P  E

      3910                3930                3950
       .                   .                   .
GCGTGTTCCGGTCGGCAAACAGATCGTCGCTCGCGCCAACCAGTCCCGCAAGGACTACGC
CGCACAAGGCCAGCCGTTTGTCTAGCAGCGAGCGCGGTTGGTCAGGGCGTTCCTGATGCG
 R  V  P  V  G  K  Q  I  V  A  R  A  N  Q  S  R  K  D  Y  A

      3970                3990                4010
       .                   .                   .
CGGGCTGCGCGAATGGTTCGATCACGAGAGCGACGACCCGGTCGCCGCCGGCCTGGCAAA
GCCCGACGCGCTTACCAAGCTAGTGCTCTCGCTGCTGGGCCAGCGGCGGCCGGACCGTTT
 G  L  R  E  W  F  D  H  E  S  D  D  P  V  A  A  G  L  A  K

      4030                4050                4070
       .                   .                   .
GTTGAAGGAACCCTCGTCCGAAGGTGTTGCTCTGCGTGAGCGGCTGTACGAGGCGCTGGA
CAACTTCCTTGGGAGCAGGCTTCCACAACGAGACGCACTCGCCGACATGCTCCGCGACCT
 L  K  E  P  S  S  E  G  V  A  L  R  E  R  L  Y  E  A  L  E

      4090                4110                4130
       .                   .                   .
GGTGAAGAACGCCGAATTCAACGCCCAGGGCGTCGAACTCAACCAGCGCTACACCTCGTC
CCACTTCTTGCGGCTTAAGTTGCGGGTCCCGCAGCTTGAGTTGGTCGCGATGTGGAGCAG
 V  K  N  A  E  F  N  A  Q  G  V  E  L  N  Q  R  Y  T  S  S

      4150                4170                4190
       .                   .                   .
CGCGGTCGTTCCCGACCCCGAGGCGGGCGAGGAAGTGTGGGTGCGCGATCGTGAGCTGTA
GCGCCAGCAAGGGCTGGGGCTCCGCCCGCTCCTTCACACCCACGCGCTAGCACTCGACAT
 A  V  V  P  D  P  E  A  G  E  E  V  W  V  R  D  R  E  L  Y

      4210                4230                4250
       .                   .                   .
CCTGCAGGCCACCACCCGGCCGGGCGCGAAGCTGCCGCATGCGTGGCTGGTCGGCGCCGA
GGACGTCCGGTGGTGGGCCGGCCCGCGCTTCGACGGCGTACGCACCGACCAGCCGCGGCT
 L  Q  A  T  T  R  P  G  A  K  L  P  H  A  W  L  V  G  A  D

      4270                4290                4310
       .                   .                   .
CGGAACCCGCATCTCCACCCTCGACGTCACCGGCAAGGGAATGATGACCCTGCTGACCGG
GCCTTGGGCGTAGAGGTGGGAGCTGCAGTGGCCGTTCCCTTACTACTGGGACGACTGGCC
 G  T  R  I  S  T  L  D  V  T  G  K  G  M  M  T  L  L  T  G

      4330                4350                4370
       .                   .                   .
ACTCGGCGGCCAGGCATGGAAGCGTGCCGCCGCCAAACTCGACCTGCCGTTCCTGCGGAC
TGAGCCGCCGGTCCGTACCTTCGCACGGCGGCGGTTTGAGCTGGACGGCAAGGACGCCTG
 L  G  G  Q  A  W  K  R  A  A  A  K  L  D  L  P  F  L  R  T

      4390                4410                4430
       .                   .                   .
CGTCGTTGTCGGCGAACCCGGCACCATCGACCCTTACGGATACTGGCGGCGGGTCCGCGA
GCAGCAACAGCCGCTTGGGCCGTGGTAGCTGGGAATGCCTATGACCGCCGCCCAGGCGCT
 V  V  V  G  E  P  G  T  I  D  P  Y  G  Y  W  R  R  V  R  D

      4450                4470                4490
       .                   .                   .
CATCGACGAGGCCGGCGCCCTGCTCGTGCGGCCCGACGGCTACGTCGCGTGGCGACACAG
```

```
GTAGCTGCTCCGGCCGCGGGACGAGCACGCCGGGCTGCCGATGCAGCGCACCGCTGTGTC
 I  D  E  A  G  A  L  L  V  R  P  D  G  Y  V  A  W  R  H  S

      4510                4530                4550

TGCTCCGGTCTGGGACGACACCGAAGCGCTCACCAGCCTCGAGAACGCTCTCACCGCGGT
ACGAGGCCAGACCCTGCTGTGGCTTCGCGAGTGGTCGGAGCTCTTGCGAGAGTGGCGCCA
 A  P  V  W  D  D  T  E  A  L  T  S  L  E  N  A  L  T  A  V

      4570                4590                4610

CCTCGACCACTCGGCCAGCGACAACGGGAACCCGAGCGGCACAAACGAGCCGCAGTACAG
GGAGCTGGTGAGCCGGTCGCTGTTGCCCTTGGGCTCGCCGTGTTTGCTCGGCGTCATGTC
 L  D  H  S  A  S  D  N  G  N  P  S  G  T  N  E  P  Q  Y  S

      4630                4650                4670

CACCCGGGCCGTGCCGATCGTCGTTCCGCACGTTACCGCCGAGGATGCAGCACCAGCTTC
GTGGGCCCGGCACGGCTAGCAGCAAGGCGTGCAATGGCGGCTCCTACGTCGTGGTCGAAG
 T  R  A  V  P  I  V  V  P  H  V  T  A  E  D  A  A  P  A  S

      4690                4710                4730

CGCCACCCGCACCACCACAGTCGAGGGAGAGAACCGATGACCCGTCCTTACACCAGCGTC
GCGGTGGGCGTGGTGGTGTCAGCTCCCTCTCTTGGCTACTGGGCAGGAATGTGGTCGCAG
 A  T  R  T  T  T  V  E  G  E  N  R  *
                                       M  T  R  P  Y  T  S  V

      4750                4770                4790

TGGGACGACCTGAACCAGGTCGAGTTCAGCCAGGGATTCATCCAGGCCGGCCCCTACCGG
ACCCTGCTGGACTTGGTCCAGCTCAAGTCGGTCCCTAAGTAGGTCCGGCCGGGGATGGCC
 W  D  D  L  N  Q  V  E  F  S  Q  G  F  I  Q  A  G  P  Y  R

      4810                4830                4850

ACCCGATACCTGCACGCCGGCGATTCGTCCAAGCCCACGCTGATCCTGCTGCACGGCATC
TGGGCTATGGACGTGCGGCCGCTAAGCAGGTTCGGGTGCGACTAGGACGACGTGCCGTAG
 T  R  Y  L  H  A  G  D  S  S  K  P  T  L  I  L  L  H  G  I

      4870                4890                4910

ACCGGCCACGCCGAGGCGTACGTGCGCAATCTGCGCTCGCATTCCGAGCACTTCAACGTC
TGGCCGGTGCGGCTCCGCATGCACGCGTTAGACGCGAGCGTAAGGCTCGTGAAGTTGCAG
 T  G  H  A  E  A  Y  V  R  N  L  R  S  H  S  E  H  F  N  V

      4930                4950                4970

TGGGCAATCGACTTCATCGGCCACGGCTATTCGACCAAGCCCGACCACCCGCTCGAGATC
ACCCGTTAGCTGAAGTAGCCGGTGCCGATAAGCTGGTTCGGGCTGGTGGGCGAGCTCTAG
 W  A  I  D  F  I  G  H  G  Y  S  T  K  P  D  H  P  L  E  I

      4990                5010                5030

AAGCACTACATCGACCACGTGCTGCAGTTGCTGGACGCCATCGGCGTCGAGAAGGCCTCG
TTCGTGATGTAGCTGGTGCACGACGTCAACGACCTGCGGTAGCCGCAGCTCTTCCGGAGC
 K  H  Y  I  D  H  V  L  Q  L  L  D  A  I  G  V  E  K  A  S

      5050                5070                5090

TTTTCCGGGGAGTCTCTCGGCGGTTGGGTCACCGCCCAGTTCGCGCACGACCATCCCGAG
AAAAGGCCCCTCAGAGAGCCGCCAACCCAGTGGCGGGTCAAGCGCGTGCTGGTAGGGCTC
 F  S  G  E  S  L  G  G  W  V  T  A  Q  F  A  H  D  H  P  E
```

```
      5110                5130                5150
       .                   .                   .                   .
AAGGTCGACCGGATCGTGCTCAACACCATGGGCGGCACCATGGCCAACCCTCAGGTGATG
TTCCAGCTGGCCTAGCACGAGTTGTGGTACCCGCCGTGGTACCGGTTGGGAGTCCACTAC
K  V  D  R  I  V  L  N  T  M  G  G  T  M  A  N  P  Q  V  M

      5170                5190                5210
       .                   .                   .                   .
GAACGTCTCTATACCCTGTCGATGGAAGCGGCGAAGGACCCGAGCTGGGAACGCGTCAAA
CTTGCAGAGATATGGGACAGCTACCTTCGCCGCTTCCTGGGCTCGACCCTTGCGCAGTTT
E  R  L  Y  T  L  S  M  E  A  A  K  D  P  S  W  E  R  V  K

      5230                5250                5270
       .                   .                   .                   .
GCACGCCTCGAATGGCTCATGGCCGACCCGACCATGGTCACCGACGACCTGATCCGCACC
CGTGCGGAGCTTACCGAGTACCGGCTGGGCTGGTACCAGTGGCTGCTGGACTAGGCGTGG
A  R  L  E  W  L  M  A  D  P  T  M  V  T  D  D  L  I  R  T

      5290                5310                5330
       .                   .                   .                   .
CGCCAGGCCATCTTCCAGCAGCCGGATTGGCTCAAGGCCTGCGAGATGAACATGGCACTG
GCGGTCCGGTAGAAGGTCGTCGGCCTAACCGAGTTCCGGACGCTCTACTTGTACCGTGAC
R  Q  A  I  F  Q  Q  P  D  W  L  K  A  C  E  M  N  M  A  L

      5350                5370                5390
       .                   .                   .                   .
CAGGACCTCGAAACCCGCAAGCGGAACATGATCACCGACGCCACTCTCAACGGCATCACG
GTCCTGGAGCTTTGGGCGTTCGCCTTGTACTAGTGGCTGCGGTGAGAGTTGCCGTAGTGC
Q  D  L  E  T  R  K  R  N  M  I  T  D  A  T  L  N  G  I  T

      5410                5430                5450
       .                   .                   .                   .
GTGCCCGCGATGGTGCTGTGGACCACCAAGGACCCCTCCGGTCCGGTCGACGAAGCCAAG
CACGGGCGCTACCACGACACCTGGTGGTTCCTGGGGAGGCCAGGCCAGCTGCTTCGGTTC
V  P  A  M  V  L  W  T  T  K  D  P  S  G  P  V  D  E  A  K

      5470                5490                5510
       .                   .                   .                   .
CGCATCGCCTCCCACATCCCGGGCGCCAAGCTGGCCATCATGGAGAACTGTGGCCACTGG
GCGTAGCGGAGGGTGTAGGGCCCGCGGTTCGACCGGTAGTACCTCTTGACACCGGTGACC
R  I  A  S  H  I  P  G  A  K  L  A  I  M  E  N  C  G  H  W

      5530                5550                5570
       .                   .                   .                   .
CCCCAGTACGAGGACCCCGAGACCTTCAACAAGCTGCATCTGGACTTCCTCCTCGGTCGC
GGGGTCATGCTCCTGGGGCTCTGGAAGTTGTTCGACGTAGACCTGAAGGAGGAGCCAGCG
P  Q  Y  E  D  P  E  T  F  N  K  L  H  L  D  F  L  L  G  R

      5590                5610                5630
       .                   .                   .                   .
AGCTGACACAGACCCCGGCCGGTGCCGCCAACCCCTGCAACCCGGGCGGCACCGGCCGGA
TCGACTGTGTCTGGGGCCGGCCACGGCGGTTGGGGACGTTGGGCCCGCCGTGGCCGGCCT
S  *

      5650                5670                5690
       .                   .                   .                   .
TCTCACTTACCCGACCTATTGCGCTCTCGTCCGGACCCCCGGAGAGAAAGCGCCGAAGCA
AGAGTGAATGGGCTGGATAACGCGAGAGCAGGCCTGGGGGCCTCTCTTTCGCGGCTTCGT

      5710                5730                5750
       .                   .                   .                   .
GCAGCAAGGAGACCGCCGCGATGCCTGTAGCGCTGTGCGCGATGTCGCACTCCCCCCTGA
```

```
CGTCGTTCCTCTGGCGGCGCTACGGACATCGCGACACGCGCTACAGCGTGAGGGGGGACT
                    M  P  V  A  L  C  A  M  S  H  S  P  L  M

      5770                5790                5810          -
       .                   .                   .            .
TGGGACGCAACGACCCCGAACAGGAAGTCATCGACGCCGTCGACGCCGCATTCGACCACG
ACCCTGCGTTGCTGGGGCTTGTCCTTCAGTAGCTGCGGCAGCTGCGGCGTAAGCTGGTGC
  G  R  N  D  P  E  Q  E  V  I  D  A  V  D  A  A  F  D  H  A

      5830                5850                5870
       .                   .                   .            .
CGCGCCGGTTCGTCGCCGACTTCGCCCCCGATCTCATCGTCATCTTCGCCCCCGACCACT
GCGCGGCCAAGCAGCGGCTGAAGCGGGGGCTAGAGTAGCAGTAGAAGCGGGGGCTGGTGA
  R  R  F  V  A  D  F  A  P  D  L  I  V  I  F  A  P  D  H  Y

      5890                5910                5930
       .                   .                   .            .
ACAACGGCGTCTTCTACGACCTGCTGCCGCCGTTCTGTATCGGTGCCGCCGCGCAGTCCG
TGTTGCCGCAGAAGATGCTGGACGACGGCGGCAAGACATAGCCACGGCGGCGCGTCAGGC
  N  G  V  F  Y  D  L  L  P  P  F  C  I  G  A  A  A  Q  S  V

      5950                5970                5990
       .                   .                   .            .
TCGGCGACTACGGCACCGAAGCCGGCCCTCTCGACGTCGACCGTGACGCCGCCTACGCAG
AGCCGCTGATGCCGTGGCTTCGGCCGGGAGAGCTGCAGCTGGCACTGCGGCGGATGCGTC
  G  D  Y  G  T  E  A  G  P  L  D  V  D  R  D  A  A  Y  A  V

      6010                6030                6050
       .                   .                   .            .
TCGCCCGCGACGTCCTCGACAGCGGCATCGACGTCGCATTCTCCGAACGCATGCACGTCG
AGCGGGCGCTGCAGGAGCTGTCGCCGTAGCTGCAGCGTAAGAGGCTTGCGTACGTGCAGC
  A  R  D  V  L  D  S  G  I  D  V  A  F  S  E  R  M  H  V  D

      6070                6090                6110
       .                   .                   .            .
ACCACGGATTCGCCCAAGCACTCCAATTGCTGGTCGGATCGATCACCGCCGTGCCGACCG
TGGTGCCTAAGCGGGTTCGTGAGGTTAACGACCAGCCTAGCTAGTGGCGGCACGGCTGGC
  H  G  F  A  Q  A  L  Q  L  L  V  G  S  I  T  A  V  P  T  V

      6130                6150                6170
       .                   .                   .            .
TGCCGATCTTCATCAATTCGGTCGCCGAACCGCTCGGCCCGGTCAGCCGGGTACGGCTGC
ACGGCTAGAAGTAGTTAAGCCAGCGGCTTGGCGAGCCGGGCCAGTCGGCCCATGCCGACG
  P  I  F  I  N  S  V  A  E  P  L  G  P  V  S  R  V  R  L  L

      6190                6210                6230
       .                   .                   .            .
TCGGCGAGGCGGTCGGGCGGGCCGCTGCCAAGCTGGACAAGCGTGTGCTGTTCGTCGGAT
AGCCGCTCCGCCAGCCCGCCCGGCGACGGTTCGACCTGTTCGCACACGACAAGCAGCCTA
  G  E  A  V  G  R  A  A  A  K  L  D  K  R  V  L  F  V  G  S

      6250                6270                6290
       .                   .                   .            .
CCGGCGGCCTGTCCCACGACCCGCCGGTCCCGCAGTTCGCCACCGCGCCAGAGGAAGTGC
GGCCGCCGGACAGGGTGCTGGGCGGCCAGGGCGTCAAGCGGTGGCGCGGTCTCCTTCACG
  G  G  L  S  H  D  P  P  V  P  Q  F  A  T  A  P  E  E  V  R

      6310                6330                6350
       .                   .                   .            .
GCGAGCGGTTGATCGACGGCCGCAATCCCAGTGCCGCCGAACGTGATGCCCGCGAACAGC
CGCTCGCCAACTAGCTGCCGGCGTTAGGGTCACGGCGGCTTGCACTACGGGCGCTTGTCG
  E  R  L  I  D  G  R  N  P  S  A  A  E  R  D  A  R  E  Q  R
```

```
      6370                6390                6410
      .                   .                   .
GCGTCATCACCGCCGGGCGGGACTTCGCCGCCGGCACCGCCGCCATCCAGCCACTGAACC
CGCAGTAGTGGCGGCCCGCCCTGAAGCGGCGGCCGTGGCGGCGGTAGGTCGGTGACTTGG
  V  I  T  A  G  R  D  F  A  A  G  T  A  A  I  Q  P  L  N  P

      6430                6450                6470
      .                   .                   .
CCGAATGGGACCGGCACCTGCTCGACGTCCTCGCCTCCGGCGACCTCGAGCAGATCGACG
GGCTTACCCTGGCCGTGGACGAGCTGCAGGAGCGGAGGCCGCTGGAGCTCGTCTAGCTGC
  E  W  D  R  H  L  L  D  V  L  A  S  G  D  L  E  Q  I  D  A

      6490                6510                6530
      .                   .                   .
CGTGGACCAACGACTGGTTCGTCGAACAGGCCGGACACTCCTCCCACGAAGTGCGCACCT
GCACCTGGTTGCTGACCAAGCAGCTTGTCCGGCCTGTGAGGAGGGTGCTTCACGCGTGGA
  W  T  N  D  W  F  V  E  Q  A  G  H  S  S  H  E  V  R  T  W

      6550                6570                6590
      .                   .                   .
GGATCGCCGCGTACGCGGCAATGAGCGCCGCCGGGAAGTACCGCGTCACCTCGACCTTCT
CCTAGCGGCGCATGCGCCGTTACTCGCGGCGGCCCTTCATGGCGCAGTGGAGCTGGAAGA
  I  A  A  Y  A  A  M  S  A  A  G  K  Y  R  V  T  S  T  F  Y

      6610                6630                6650
      .                   .                   .
ACCGCGAAATCCACGAGTGGATAGCAGGATTCGGGATTACTACCGCCGTCGCCGTCGACG
TGGCGCTTTAGGTGCTCACCTATCGTCCTAAGCCCTAATGATGGCGGCAGCGGCAGCTGC
  R  E  I  H  E  W  I  A  G  F  G  I  T  T  A  V  A  V  D  E

      6670                6690                6710
      .                   .                   .
AATAGACCCCGCCGCTCCCGCCCCGCAGTCCCAACGAAGGGTGGCCCCGGATGACCTCCG
TTATCTGGGGCGGCGAGGGCGGGGCGTCAGGGTTGCTTCCCACCGGGGCCTACTGGAGGC
  *                                                 M  T  S  V

      6730                6750                6770
      .                   .                   .
TCCGCCCGTGCTCGCCGTCGGTGAACGCGGGCTGGTCGGTGGGCAGGAAGACCTCATCGC
AGGCGGGCACGAGCGGCAGCCACTTGCGCCCGACCAGCCACCCGTCCTTCTGGAGTAGCG
  R  P  C  S  P  S  V  N  A  G  W  S  V  G  R  K  T  S  S  P

      6790                6810                6830
      .                   .                   .
CGACATCGCCCTCGACCTCGCAGCTCGTCAGTAGGAATGCGCACGGGCCGACGAGTCGCG
GCTGTAGCGGGAGCTGGAGCGTCGAGCAGTCATCCTTACGCGTGCCCGGCTGCTCAGCGC
  T  S  P  S  T  S  Q  L  V  S  R  N  A  H  G  P  T  S  R  A

      6850                6870                6890
      .                   .                   .
CTGGTCACCGGGGCCAGCCGCGGCATCGGGGCGGCCATCGCAGATGCGGTGGCCGCCTCC
GACCAGTGGCCCCGGTCGGCGCCGTAGCCCCGCCGGTAGCGTCTACGCCACCGGCGGAGG
  G  H  R  G  Q  P  R  H  R  G  G  H  R  R  C  G  G  R  L  R

      6910                6930                6950
      .                   .                   .
GGTGCCGCCGTAATCGTCCACTACGGATCCGATCGGACGGCCGCCGCTGCGGTGTCGACG
CCACGGCGGCATTAGCAGGTGATGCCTAGGCTAGCCTGCCGGCGGCGACGCCACAGCTGC
  C  R  R  N  R  P  L  R  I  R  S  D  G  R  R  C  G  V  D  G

      6970                6990                7010
      .                   .                   .
GCATCACGGCTGCCGGGGGCCTCGCGGCTGCGGTCCAGGCCGACCTGTCCCGACCCGAGG
```

```
CGTAGTGCCGACGGCCCCCGGAGCGCCGACGCCAGGTCCGGCTGGACAGGGCTGGGCTCC
  I  T  A  A  G  G  L  A  A  A  A  V  Q  A  D  L  S  R  P  E  G

      7030                 7050                 7070
       .    .    .    .    .    .    .    .    .    .    .    .
GGCCTGAAGAGCTGATGCGGGAGTTCGACTCCGCGCTCGACGGTCTCGGGCTCGACCGAG
CCGGACTTCTCGACTACGCCCTCAAGCTGAGGCGCGAGCTGCCAGAGCCCGAGCTGGCTC
  P  E  E  L  M  R  E  F  D  S  A  L  D  G  L  G  L  D  R  G

      7090                 7110                 7130
       .    .    .    .    .    .    .    .    .    .    .    .
GGCTCGACATCCTCGTCAACAACGCCGGAATCAGTCGGCGCGGAGCGCTCGAGCGCGTCA
CCGAGCTGTAGGAGCAGTTGTTGCGGCCTTAGTCAGCCGCGCCTCGCGAGCTCGCGCAGT
  L  D  I  L  V  N  N  A  G  I  S  R  R  G  A  L  E  R  V  T

      7150                 7170                 7190
       .    .    .    .    .    .    .    .    .    .    .    .
CTGTCGAGGATTTCGACCGTCTGGTCGCACTCAACCAGCGCGCCCCGTTCTTCGTGACTC
GACAGCTCCTAAAGCTGGCAGACCAGCGTGAGTTGGTCGCGCGGGGCAAGAAGCACTGAG
  V  E  D  F  D  R  L  V  A  L  N  Q  R  A  P  F  F  V  T  R

      7210                 7230                 7250
       .    .    .    .    .    .    .    .    .    .    .    .
GGCATGCCCTGCCCCGGATGCACGACGGCGGTCGCATCGTCAACATTTCCTCCGGATCCG
CCGTACGGGACGGGGCCTACGTGCTGCCGCCAGCGTAGCAGTTGTAAAGGAGGCCTAGGC
  H  A  L  P  R  M  H  D  G  G  R  I  V  N  I  S  S  G  S  A

      7270                 7290                 7310
       .    .    .    .    .    .    .    .    .    .    .    .
CCCGCTACGCCAGACCCGACGTCATCAGCTACGCCATGACCAAGGGGGCGATCGAGGTGC
GGGCGATGCGGTCTGGGCTGCAGTAGTCGATGCGGTACTGGTTCCCCCGCTAGCTCCACG
  R  Y  A  R  P  D  V  I  S  Y  A  M  T  K  G  A  I  E  V  L

      7330                 7350                 7370
       .    .    .    .    .    .    .    .    .    .    .    .
TCACCCGCGCCCTCGCCGTAGACGTCGGCGAACGAGGCATCACCGCCAACGCCGTGGCGC
AGTGGGCGCGGGAGCGGCATCTGCAGCCGCTTGCTCCGTAGTGGCGGTTGCGGCACCGCG
  T  R  A  L  A  V  D  V  G  E  R  G  I  T  A  N  A  V  A  P

      7390                 7410                 7430
       .    .    .    .    .    .    .    .    .    .    .    .
CGGCCGCGCTCGATACCGACATGAACGCGCACTGGCTTCGCGGTGACGACCATGCCCGCA
GCCGGCGCGAGCTATGGCTGTACTTGCGCGTGACCGAAGCGCCACTGCTGGTACGGGCGT
  A  A  L  D  T  D  M  N  A  H  W  L  R  G  D  D  H  A  R  T

      7450                 7470                 7490
       .    .    .    .    .    .    .    .    .    .    .    .
CCACCGCCGCGTCCACCACTGCACTGCGAAAACTCGCCACCGCGGAGGACATCGCCGCGA
GGTGGCGGCGCAGGTGGTGACGTGACGCTTTTGAGCGGTGGCGCCTCCTGTAGCGGCGCT
  T  A  A  S  T  T  A  L  R  K  L  A  T  A  E  D  I  A  A  I

      7510                 7530                 7550
       .    .    .    .    .    .    .    .    .    .    .    .
TCGTGGCCTTCCTCGTCAGCGCCGCCGCCGGTGCGATCACCGGGCAGGTCATCGACGCCA
AGCACCGGAAGGAGCAGTCGCGGCGGCGGCCACGCTAGTGGCCCGTCCAGTAGCTGCGGT
  V  A  F  L  V  S  A  A  A  G  A  I  T  G  Q  V  I  D  A  T

      7570
       .    .
CCAACGGCAACCGGCTCTAACCAG
GGTTGCCGTTGGCCGAGATTGGTC
  N  G  N  R  L  *
```

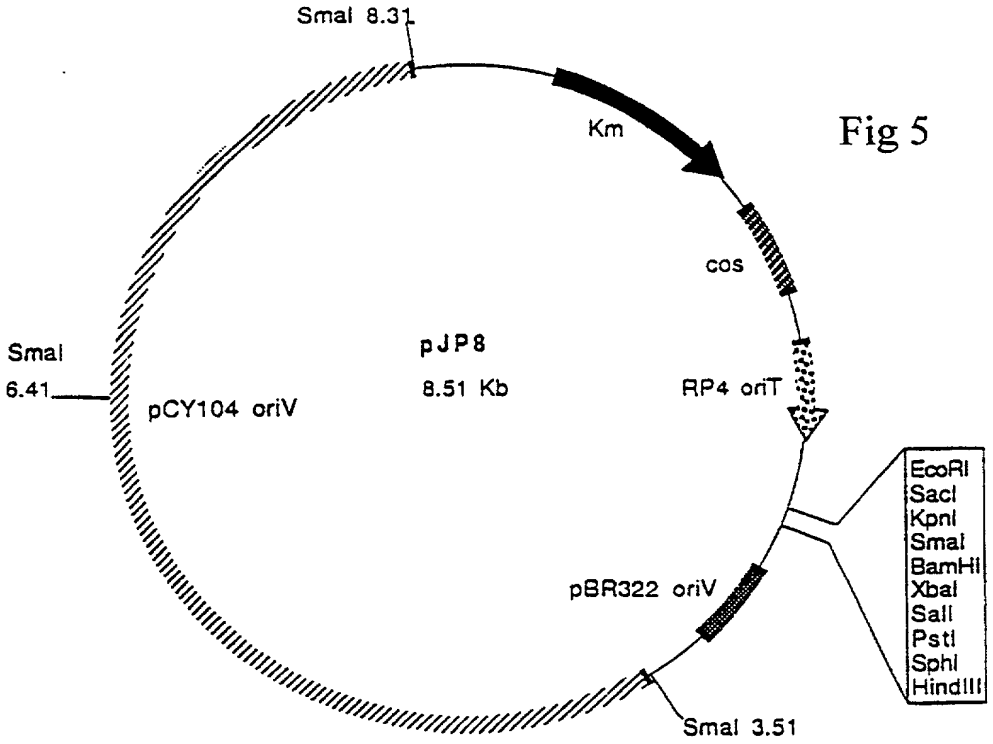

SmaI 8.31
Km
cos
SmaI
6.41
pJP8
8.51 Kb
pCY104 oriV
RP4 oriT
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
pBR322 oriV
SmaI 3.51

Fig 5

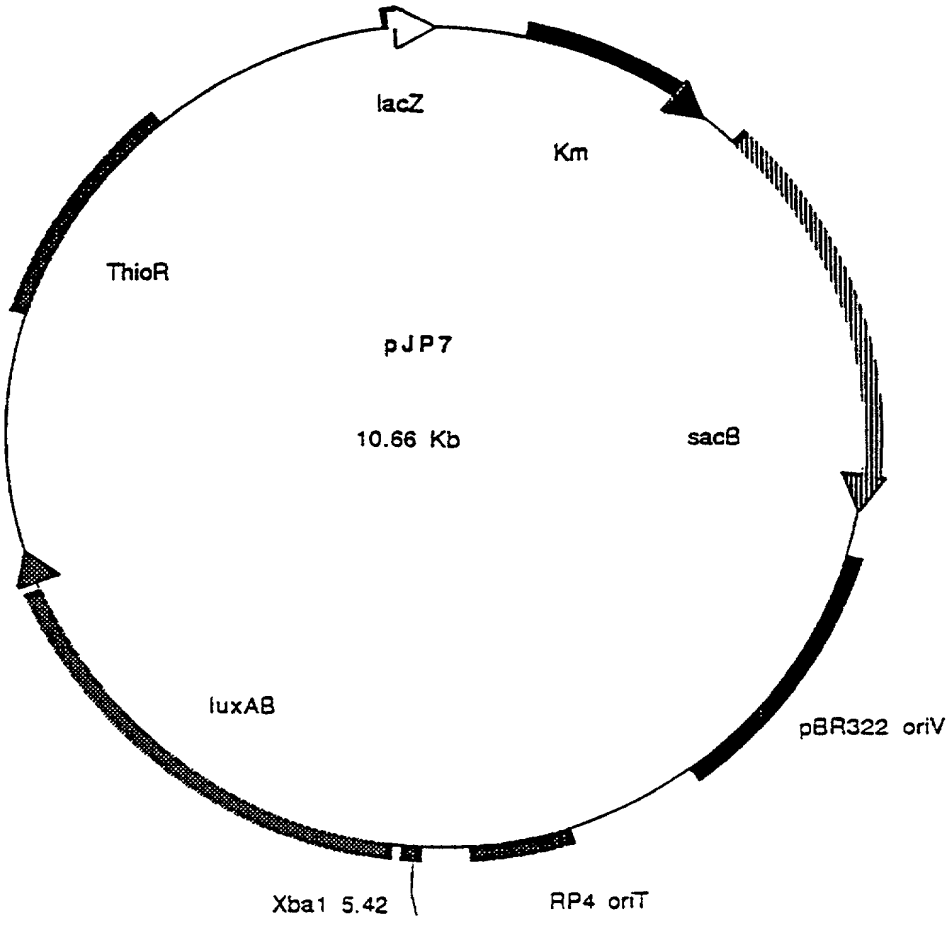

lacZ
Km
ThioR
pJP7
10.66 Kb
sacB
luxAB
pBR322 oriV
Xba1 5.42
RP4 oriT

Fig 6

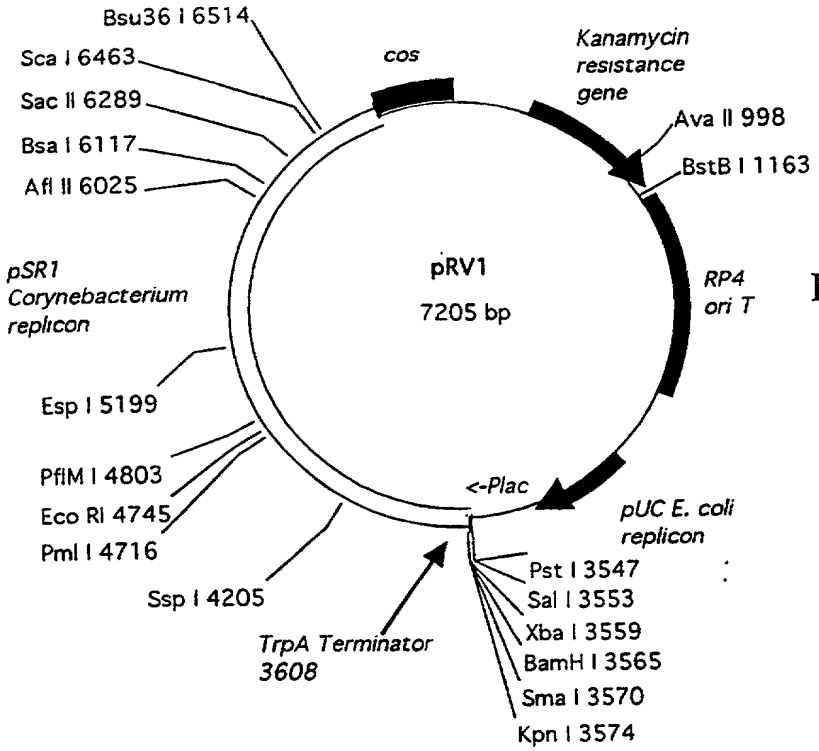

Bsu36 I 6514
Sca I 6463
Sac II 6289
Bsa I 6117
Afl II 6025
cos
Kanamycin resistance gene
Ava II 998
BstB I 1163
pSR1 Corynebacterium replicon
pRV1
7205 bp
RP4 ori T
Esp I 5199
PflM I 4803
Eco RI 4745
Pml I 4716
Ssp I 4205
<-Plac
pUC E. coli replicon
Pst I 3547
Sal I 3553
Xba I 3559
BamH I 3565
Sma I 3570
Kpn I 3574
TrpA Terminator 3608

Fig 7

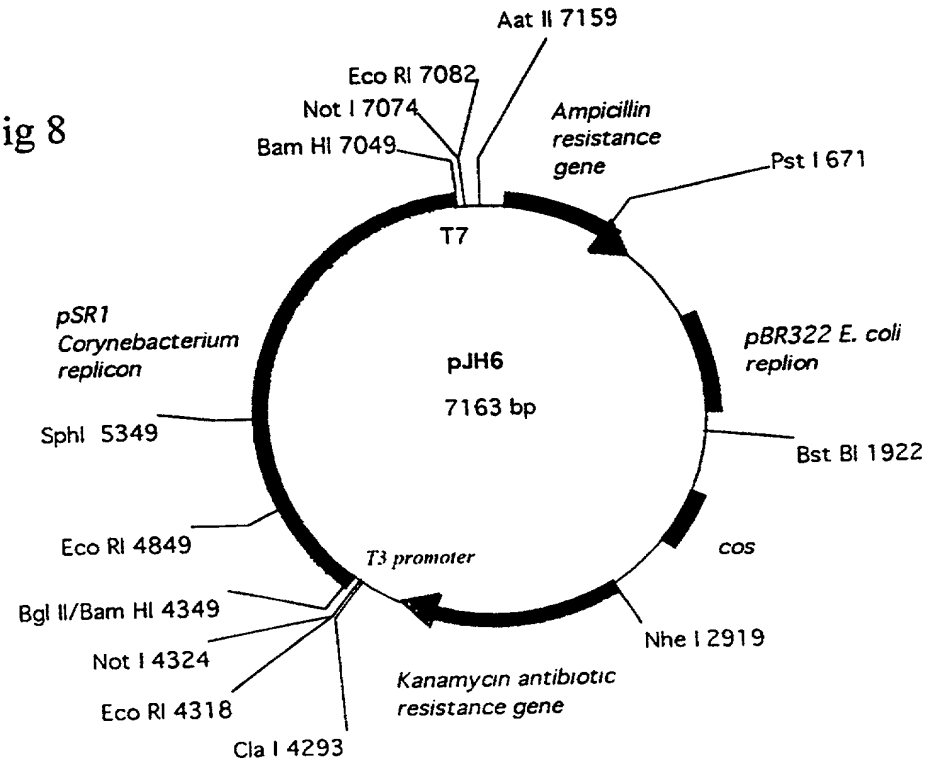

Aat II 7159
Eco RI 7082
Not I 7074
Bam HI 7049
Ampicillin resistance gene
Pst I 671
T7
pSR1 Corynebacterium replicon
pJH6
7163 bp
pBR322 E. coli replion
SphI 5349
Bst BI 1922
Eco RI 4849
T3 promoter
cos
Bgl II/Bam HI 4349
Nhe I 2919
Not I 4324
Kanamycin antibiotic resistance gene
Eco RI 4318
Cla I 4293

Fig 8

UTILITY
Original U.S. or PCT D/O
Foreign Priority

SUBSTITUTE DECLARATION, POWER OF ATTORNEY AND POWER TO INSPECT

As a below named inventor, I hereby declare:

that my residence, post office address and citizenship are as stated below next to my name;

that I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the invention entitled: **BIOSENSOR MATERIALS AND METHODS**

the specification of which [check one(s) applicable]
__X__ was filed 29 June 1998 as International Application No. PCT/GB98/01893 [now U.S. Application No. 09/446,681]
_____ and was amended by Amendment filed _____ (if applicable); [or];
_____ is attached to this Declaration, Power of Attorney and Power to Inspect;

that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and

that I acknowledge my duty to disclose information which is material to the examination of this application in accordance with Rule 56(a) [37CFR§1.56(a)].

CLAIM UNDER 35 USC §119: I hereby claim foreign priority benefits under 35 USC §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

Application No.	Country	Filing Date Day-Mo-Year	Yes - No
9713666.7	GB	27 June 1997	YES

POWER OF ATTORNEY: As inventor, I hereby appoint **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, PA, and the following individual(s) as my attorneys or agents with full power of substitution to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **Patrick J. Hagan, Reg. No. 27,643 and Kathleen D. Rigaut, Ph.D., Reg. No. 43,047.**

POWER TO INSPECT: I hereby give **DANN, DORFMAN, HERRELL AND SKILLMAN, P.C.** of Philadelphia, PA or its duly accredited representatives power to inspect and obtain copies of the papers on file relating to this application.

SEND CORRESPONDENCE TO: CUSTOMER NUMBER 000110.

DIRECT INQUIRIES TO: Patrick J. Hagan, Esq.
Telephone: (215) 563-4044
Facsimile: (215) 563-4044

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

SOLE OR FIRST JOINT INVENTOR

Full Name John (First) Anthony Charles (Middle) ARCHER (Last)
Signature John Anthony Charles Archer
Date 15th February 2000
Residence Cambridge, UK GBX
(City) (State or Country)
Citizenship British
Post Office Address:
36 West Field Little Abington
Cambridge Cambridgeshire CB6 1BE
(City) (State or Country) (Zip Code)

2-00

SECOND JOINT INVENTOR (IF ANY)

Full Name David (First) Keith (Middle) SUMMERS (Last)
Signature [signature]
Date 15th Feb. 2000
Residence CAMBRIDGE, UK GBX
(City) (State or Country)
Citizenship BRITISH
Post Office Address:
34 HUMBERSTONE ROAD,
CAMBRIDGE, CAMBS., CB4 1JF
(City) (State or Country) (Zip Code)

3-00

THIRD JOINT INVENTOR (IF ANY)

Full Name Herve (First) Jacquiau (Middle) ROLAND (Last)

Signature [signature]

Date 15 February 2000

Residence Cambridge (City) England (State or Country) ENG

Citizenship French

Post Office Address:

23 Callander Close

Cambridge England CB4 2XJ
City State or Country Zip Code

4-00

FOURTH JOINT INVENTOR (IF ANY)

Full Name Justin (First) Antoine Christian (Middle) POWELL (Last)

Signature Justin Antoine Christian Powell

Date 15 February 2000

Residence Cambridge (City) ENGLAND (State or Country) ENG

Citizenship BRITISH

Post Office Address:

38 De Freville Ave

CAMBRIDGE ENGLAND CB4 1HT
City State or Country Zip Code